Development of Novel Hydrogen sulfide Therapeutics for Translation to the Clinic

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Hydrogen sulfide (H_2S) based therapeutics have emerged as a viable candidate for the treatment of a number of cardiovascular diseases. At present there are numerous reports demonstrating potent effects of H_2S donors and releasing agents in preclinical models of acute myocardial infarction, heart failure, peripheral vascular disease and cerebrovascular diseases. While these studies support the clinical translation of H_2S therapeutics for cardiovascular disease there are a number of issues that must be addressed for successful transition from bench to bedside. One of the most significant limitations for translation of H_2S therapeutics is the lack of H_2S-donors with suitable pharmacokinetic profiles for the use in chronic cardiovascular diseases. Most of the currently available H_2S donor agents release H_2S in a rapid and uncontrolled manner and this can result in poor efficacy or even toxicity if H_2S levels rise to suprapharmacological levels. Data will be presented describing novel H_2S donors that are engineered for controlled and sustained H_2S release under in vivo pathological conditions. Another significant hurdle that must be overcome for the development of H_2S to treat cardiovascular disease is a more complete understanding of the role(s) of the 3 endogenous enzymes cystathionine gamma lyase (CSE), cystathionine beta synthase (CBS), and 3-mercaptopyruvate sulfur transferase (3-MST) in the physiology and pathology of the cardiovascular system. Data will be presented describing the roles of various H_2S-generating enzymes in cardiovascular disease states. Another requirement for clinical translation of H_2S to cardiovascular disease in man is the testing of H_2S in more rigorous, clinically relevant, large animal models of cardiovascular disease. To date the majority of published reports of the cardioprotective and vasculoprotective effects of H_2S involve rodent models of disease.

These studies represent a strong first step, but confirmation of these beneficial effects in large animal models is necessary prior to the initiation of clinical studies in cardiovascular disease. Data will be presented describing the results of recent experiments in the area of peripheral vascular disease (i.e., critical limb ischemia) in a porcine model that very closely mimics the human clinical condition. While H_2S therapies present a viable and exciting drug class for the treatment of acute and chronic cardiovascular diseases there is still remaining work to be done to ensure successful translation to the clinic.

Preventing Myocardial Ischemia-Reperfusion Injury and Failure with Hydrogen sulfide

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Hydrogen sulfide (H_2S) has been long recognized as a highly poisonous gas that is rapidly lethal in intoxicating dosage. However, discoveries during the last decade on the endogenous synthesis of H2S in the mammalian system and its protective role in combating cellular necrosis, apoptosis, oxidative stress, inflammation as well as promoting angiogenesis and modulation of mitochondrial respiration in the setting of myocardial ischemia and reperfusion injury have prompted vast interest in the possibility of developing new therapies based around mimicry or facilitation of endogenous H_2S for cardioprotection (1,5,7). These observations have inspired rapid development of H2S-releasing drugs in hopes of swift clinical translation in patients with cardiovascular disease. Our initial studies were focused on investigating the role of H_2S in mediating the cardioprotective effects of the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG) axis (2,3,9), which were later expanded to encompass the mechanistic effects of H_2S-donor therapy in the context of miRNA modulation and mitigation of cardiac inflammasome formation/activation post myocardial infarction (6). We further identified novel protein targets, using proteomics, that may play key roles in the evolution of heart failure secondary to myocardial infarction and we are currently studying the impact of H_2S therapy on these proteins [mitochondrial antiviral signaling (MAVS) and cofilin-2 in the context of acute myocardial infarction and heart failure. MAVS has been implicated in attenuating bax-mediated cytochrome release following oxidative stress in cardiomyocytes (4) and cofilin-2 expression has been shown to increase and form aggregates in the hearts of patients with idiopathic dilated cardiomyopathy (8). Our new results show that H_2S treatment by way of donors fails to attenuate myocardial infarction in MAVS knockout mice and we also have preliminary data that demonstrate increased expression of cardiac cofilin-2 in patients with end-stage heart failure

concomitant with decreased expression of cardiac cystathionine-γ-lyase as compared to control hearts. We hope that more discoveries will be made to illustrate the versatile benefits of H_2S and its potential for clinical translation in patients with cardiovascular disease and heart failure.

References:

1. Elrod J.W., Calvert J.W., Morrison J., Doeller J.E., Kraus D.W., Tao L., Jiao X., Scalia R., Kiss L., Szabo C., Kimura H., Chow C.W., Lefer D.J. Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. Proc Natl Acad Sci USA. 2007;104:15560-5.

2. Salloum F.N., Chau V.Q., Hoke N.N., Abbate A., Varma A., Ockaili R.A., Toldo S., Kukreja R.C. Phosphodiesterase-5 Inhibitor, Tadalafil, Protects against Myocardial Ischemia/Reperfusion through Protein-Kinase G Dependent Generation of Hydrogen Sulfide. Circulation. 2009;120:S31-6.

3. Salloum F.N., Das A., Samidurai A., Hoke N.N., Chau V.Q., Ockaili R.A., Stasch J.P., Kukreja R.C. Cinaciguat - a novel activator of soluble guanylate cyclase, protects against ischemia/reperfusion injury: Role of hydrogen sulfide. Am J Physiol Heart Circ Physiol. 2012;302:H1347-54.

4. Toshitaka Yajima, Stanley Park, Hanbing Zhou, Michinari Nakamura, Mitsuyo Machida, Arthur Uyesugi, Vineel Pokala, and Andrea Domenighetti. Mitochondrial Antiviral Signaling (MAVS) Protects Cardiomyocytes Under Oxidative Stress by Interfering with Bax-Mediated Cell Death. Circ Res. 2012;111:A371.

5. Polhemus D.J. and Lefer D.J. Emergence of hydrogen sulfide as an endogenous gaseous signaling molecule in cardiovascular disease. Circ Res. 2014;114:730-737.

6. Toldo S., Das A., Mezzaroma E., Chau V.Q., Marchetti C., Durrant D., Samidurai A., Van Tassell B.W., Yin C., Ockaili R.A., Vigneshwar N., Mukhopadhyay N.D., Kukreja R.C., Abbate A., Salloum F.N. Induction of MicroRNA-21 with Exogenous Hydrogen Sulfide Attenuates Myocardial Ischemic and Inflammatory Injury in Mice. Circ Cardiovasc Genet. 2014;7:311-20.

7. Salloum F.N. Hydrogen sulfide and cardioprotection – Mechanistic insights and clinical translatability. Pharmacol Ther. 2015; 152:11-17.

8. Subramanian K., Gianni D., Balla C., Assenza G.E., Joshi M., Semigran M.J., Macgillivray T.E., Van Eyk J.E., Agnetti G., Paolocci N., Bamburg J.R., Agrawal P.B., del Monte F. Cofilin-2 phosphorylation and sequestration in myocardial aggregates: Novel pathogenic mechanisms for idiopathic dilated cardiomyopathy. J Am Coll Cardiol. 2015;65:1199-214.

9. Das A., Samidurai A., Hoke N.N., Kukreja R.C., Salloum FN. Hydrogen Sulfide Mediates the Cardioprotective Effects of Gene Therapy with PKGIα. Basic Res Cardiol. 2015;110:42.

Sulfide Biogenesis and Oxidation

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Despite the excitement about the varied physiological effects mediated by H_2S and the consequent profusion of literature on H_2S biology, there is a large gap in our understanding of how cells maintain very low steady-state levels of H_2S and amplify the signal as needed (1). Three enzymes in the sulfur network are important for its biogenesis. Two catalyze well-described non-H_2S producing reactions in the transsulfuration pathway and also synthesize cysteine persulfide from cystine (2), raising questions about how the decision between these competing reactions is made in the cell. The pathway for H_2S oxidation resides in the mitochondrion where the enzymes successively oxidize sulfide to sulfate. While sulfate is innocuous, a number of the intermediates in the sulfide oxidation pathway are reactive and their role in sulfide-based signaling remains to be assessed (3).

We have recently discovered a noncanonical sulfide oxidation pathway in red blood cells, which lack mitochondria and will discuss the challenging heme-dependent oxidation chemistry that it uses (4).

References:

1. Kabil O., Banerjee R. The redox biochemistry of hydrogen sulfide. J Biol Chem. 2010;285:21903-21907.

2. Yadav P.K. et al. Biosynthesis and Reactivity of Cysteine Persulfides in Signaling. J Am Chem. 2016;138:289-299.

3. Mishanina T.V., Libiad M., Banerjee R. Biogenesis of reactive sulfur species for signaling by hydrogen sulfide oxidation pathways. Nat Chem Biol. 2015;11:457-464.

4. Vitvitsky V., Yadav P.K., Kurthen A., Banerjee R. Sulfide oxidation by a noncanonical pathway in red blood cells generates thiosulfate and polysulfides. J Biol Chem 2015. 290:8310-8320.

Sources, targets and pathways: an update of Hydrogen sulfide Signaling

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Once viewed exclusively as an environmental pollutant and toxicant, H_2S is now recognized as an endogenous biological mediator with important roles in physiology and disease. H_2S is generated both through enzymatic and non-enzymatic pathways. Three enzymes are known to be involved in catalytic reactions the yield H_2S. Two of them cystathionine beta synthase (CBS) and cystathionine gamma lyase (CSE) are part of the trans-sulfuration pathway and use L-cysteine as a substrate to produce H_2S. The third enzyme is 3-mercaptopyruvate sulfurtransferase (3-MST) and converts 3-mercaptopyruvate (3-MP) to H_2S and pyruvate. CBS is allosterically activated by S-adenosylomethionine and is unique among PLP-dependent enzymes as it is the one and only known to carry a heme prosthetic group. 3MST differs from CSE and CBS in co-factor requirement as it does not need PLP for its catalytic activity, and is found in both cytosolic and mitochondrial fractions of cells. In spite of intensive research in the field, it is still unclear how minute-to-minute regulation of H_2S levels is achieved, as would be expected for any signaling molecule. Although Ca2+/calmodulin were initially reported to stimulate CSE activity, this effect is minimal and its biological importance remains to be established. More recently, CSE and CBS were shown to be phosphorylated and CBS is glutathionylated; all of these post translational modifications impact on enzymatic activity. A major mechanism through which H_2S signals in mammalian cells is persulfidation; examples of this mode of action for H_2S include the activation of NFκB, glyceraldehyde phosphate dehydrogenase and ATP-sensitive potassium channels. H_2S also activates kinases, inhibits phosphatases, alters the activity of ion channels and enhances the activity of transcription factors like Nrf-2 and HIF-1. In addition, H_2S exhibits both direct and indirect anti-oxidant properties. In the cardiovascular system H_2S is known to exert

a variety of beneficial effects; these include vasorelaxation, cardioprotection, and reduced atherosclerosis development. Nitric oxide plays a prominent role in H_2S-triggered responses in the cardiovascular system, as genetic deletion or pharmacological inhibition of eNOS inhibits the ability of H_2S to relax blood vessels, to enhance angiogenesis and to protect the heart. H_2S also impacts on cellular bioenergetics and can be used as inorganic fuel to preserve electron flow and sustain ATP production, which might become important when Krebs cycle-derived electron donors are insufficient to cover cellular bioenergetic demands. Given the pleiotropic effects of H_2S and its deregulation in several states disease, efforts are underway to translate basic science discoveries into clinically useful treatments. Pharmacological agents to inhibit excessive production of H_2S, and H_2S donors to restore low levels where needed, are currently being evaluated in preclinical and clinical models.

Free Thiols and Gaseous Signalling Molecules in Health and Disease

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Oxidative stress is cardinal in the pathophysiology of aging and diseases. As free thiols are readily oxidized by reactive oxygen and sulfur species, their circulating level may directly reflect the overall redox status. This is of particular interest since free thiols are amenable to therapeutic modulation. Reversible oxidative posttranslational modifications of protein thiols by several small molecules such as the gaseous signaling molecules nitric oxide and hydrogen sulfide (H_2S) have been suggested to protect proteins from irreversible oxidative damage. The modifications these molecules induce, individually or in conjunction, are S-nitrosylation, and S-sulfhydration. S-sulfhydration is indirectly brought about by H_2S, either through reactions of oxidized thiols with sulfide or through reactions of sulfide oxidation products (including polysulfides) with thiols, giving rise to persulfides. Pharmacological stimulation of the molecules inducing these reversible protein modifications could be a strategy to influence the amount of free thiols. Certain thiols are involved in redox signaling by acting as redox-switches and oxidative posttranslational modification of these critical protein thiols can alter protein function.

Our current studies attempt to elucidate whether the overall system architecture including its regulatory elements can be interrogated by evaluating the system in action using healthy human conditions and different perturbations such as human disease settings.

For that purpose we studied free thiols and end-products of hydrogen sulfide i.e. sulfate and thiosulfate in healthy individuals (n=6855), renal transplant patients (n=707) (1), healthy living donors before and after donation (n=110), type II diabetic patients (n=1004) (2), and patients with heart failure (n=101) (3).

References:

1. van den Berg E., Pasch A., Westendorp W.H., Navis G., Brink E.J., Gans R.O., van Goor H., Bakker S.J. Urinary sulfur metabolites associate with a favorable cardiovascular risk profile and survival benefit in renal transplant recipients. J Am Soc Nephrol. 2014;25(6):1303-12.

2. van den Born J.C., Frenay A.R., Bakker S.J., Pasch A., Hillebrands J.L., Lambers Heerspink H.J., van Goor H. High urinary sulfate concentration is associated with reduced risk of renal disease progression in type 2 diabetes. Nitric Oxide. 2016;55-56:18-24.

3. van Goor H., van den Born J.C., Hillebrands J.L., Joles J.A. Hydrogen sulfide in hypertension. Curr Opin Nephrol Hypertens. 2016;25(2):107-13.

4. van den Born J.C., Hammes H.P., Greffrath W., van Goor H., Hillebrands J.L., DFG GRK International Research Training Group 1874 Diabetic Microvascular Complications (DIAMICOM). Gasotransmitters in Vascular Complications of Diabetes. Diabetes. 2016;65(2):331-45.

Production and function of Hydrogen sulfide and Hydrogen polysulfide

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Two decades have passed since the first identification of endogenous H_2S in the mammalian brain, and studies of this molecule uncovered physiological roles in processes such as neuromodulation, vascular tone regulation, cytoprotection against and oxidative stress. We previously demonstrated that H_2S induces Ca^{2+} influx in astrocytes by activating transient receptor potential (TRP) channels, and during this study we found that H_2S_n activates TRP channels much more potently than does H_2S (1-2). We recently found H_2S_n in the brain and that it activates TRP ankyrin 1 channels (3). Subsequently, several other effects of H_2S_n have been reported; suppression of the tumor suppressor phosphatase and tensin homolog, facilitation of the translocation of nuclear factor-like 2 to the nucleus to upregulate antioxidant genes, the reduction of blood pressure by dilating vascular smooth muscle though the activation of protein kinase G, and facilitation of neuronal differentiation. Despite these important roles of H_2S_n, the number of sulfur atoms in H_2S_n and whether they are produced in cells were unknown. We recently identified H_2S_3 as an important H_2S_n in the brain and its producing enzyme 3-mercaptopyruvate sulfurtransferase (4).

References:

1. Nagai et al. Polysulfides induce calcium waves in rat hippocampal astrocytes. J. Pharmacol. Sci. 2006 100: 200.

2. Oosumi et al. Polysulfide activates TRP channels and increases intracellular Ca^{2+} in astrocytes. Neurosci. Res. 2010;685, e109-e222.

3. Kimura et al. Polysulfides are possible H_2S-derived signaling molecules in rat brain. FASEB J. 2013;27:2451-2457.

4. Kimura et al. Identification of H_2S_3 and H_2S produced by 3-mercaptopyruvate sulfurtransferase in the brain. Sci. Rep. 2015;5:14774.

Well Beyond a Simple 'Cross-Talk' Story: On the Unique Position of Reactive Sulfur Species in the Redox Metabolome

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The development of life on Earth was intimately linked to the composition of Earth's primordial atmosphere, the availability of redox-active metals (enabling electron transfer processes), and other inorganic soil constituents that could be exploited as nutrients. While little is known about the earliest life forms and few fossil records from this time have survived to current study, several lines of evidence suggest that the early atmosphere on Earth was essentially anoxic and sulfidic (i.e. containing hydrogen sulfide, H_2S) (1). Sulfide became used as a substrate for energy production by monocellular sulfoxidizing organisms that later developed into mitochondrial organelles, fuelling much of the metabolic machinery of contemporary eukaryotic cells. In order to survive and thrive those early life forms must have required elaborate sensing and response elements to appropriately adjust energetic needs and metabolic pathways to changes in external conditions. We believe that sulfide played a fundamental role in accomplishing this feat, preceeding oxygen and nitric oxide (NO)-based signaling (1,2). This would explain why modulating systemic sulfide availability, either by inhibiting enzymes involved in its production or by addition of sulfide salts and H_2S-generating compounds, is accompanied by changes in local and global redox status, in turn affecting a large number of other redox-linked cell signaling processes. This presentation will discuss the role sulfur is likely to have played in shaping intermediary metabolism and redox signaling and explain why it continues to play rather fundamental functions in regulating cellular and bodily processes. Its role as an evolutionary early substrate and messenger helps conceptualizing the 'cross-talk' between reactive oxygen species (ROS) and NO as well as that between the NO and H_2S/polysulfide/reactive sulfur species (RSS) signaling pathways. While peroxynitrite ($ONOO^-$), a key

product of interaction of NO with superoxide, was reported to oxidize thiol groups 25 years ago, more recent investigations suggest that the sulfur analogon of peroxynitrite, nitrosopersulfide ($SSNO^-$/$ONSS^-$) - one of several reaction products of the NO/H_2S interaction - may play a similarly fundamental role in cell physiology by giving rise to polysulfide formation and by enabling sulfur trafficking, electron transfer and redox signaling (4,5). We here propose that all these reactive species are part of the same cellular redox network.

References:

1. Olson K.R., Straub K.D. The Role of Hydrogen Sulfide in Evolution and the Evolution of Hydrogen Sulfide in Metabolism and Signaling. Physiology (Bethesda). 2016;31(1):60-72.

2. Feelisch M., Martin J.F. The Early Role of Nitric Oxide in Evolution. Trends Ecol Evol 1995;10(12):496-9.

3. Radi R., Beckman J.S., Bush K.M., Freeman B.A. Peroxynitrite Oxidation of Sulfhydryls. The Cytotoxic Potential of Superoxide and Nitric Oxide. J Biol Chem 1991;266(7):4244-50.

4. Cortese-Krott M.M., Kuhnle G.G., Dyson A., Fernandez B.O., Grman M., DuMond J.F., Barrow M.P., McLeod G., Nakagawa H., Ondrias K., Nagy P., King S.B., Saavedra J.E., Keefer L.K., Singer M., Kelm M., Butler A.R., Feelisch M. Key Bioactive Reaction Products of the NO/H2S Interaction are S/N-Hybrid Species, Polysulfides, and Nitroxyl. Proc Natl Acad Sci USA. 2015;112(34):E4651-60.

5. Cortese-Krott M.M., Butler A.R., Woollins J.D., Feelisch M. Inorganic sulfur-nitrogen compounds: from gunpowder chemistry to the forefront of biological signaling. Dalton Trans 2016;45(14):5908-19.

Insights into the molecular pathways of Persulfide-mediated Redox Signaling

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Persulfide-mediated redox signaling and protection against oxidative stress are in the focus of hydrogen sulfide biology (1,2). Our research is centered on the underlying chemical basis and enzymology of persulfide formation and catabolism in a cellular context (3).

We demonstrated that inorganic polysulfides can link H_2S to protein thiol oxidation. We found that oxidation of the tumor suppressor PTEN protein by polysulfides is a highly favorable process even in a very reducing milieu, representing an efficient redox switch for the enzyme (4). We provided evidence that H_2S can react with reactive disulfide species in thiol/disulfide exchange-like reactions to produce protein persulfides and showed that in extreme situations even the transfer of the oxidizing equivalent (coming from the disulfide) to produce inorganic polysulfides can be energetically favorable (although the biological significance of these reactions remain to be demonstrated) (5). Beside our work, an increasing number of studies are now focused on the mechanisms of intracellular protein persulfide formation via enzymatic and non-enzymatic processes.

In contrast, the regeneration of protein Cys residues from persulfides, which is apparently a requirement for a bona-fide signaling model, has not been widely investigated. In our recent paper we developed a novel persulfide detection method (ProPerDP), which allowed us to undertake the first comprehensive study on the regeneration of Cys residues from persulfide species in a cellular context and *in vivo* (6). ProPerDP is specific, easy to use and utilize only commercially available material. I will demonstrate the critical steps of our method and demonstrate its utility for the sulfide community. Using an enzyme kinetics approach we demonstrated that the *thioredoxin* system is highly efficient in reducing inorganic polysulfides and

protein persulfides. We found that *thioredoxin reductase 1* (TrxR1) on its own can use NADPH to reduce sulfane sulfur species. However, inclusion of *thioredoxin 1* (Trx1), but more so the relatively newly discovered *small thioredoxin like protein of 14 kDa* (TRP14) in the enzyme kinetic assays further increased polysulfide and protein persulfide reduction rates. In model cellular systems (where different components of the Trx machinery were knocked down) ProPerDP revealed that TrxR1 and TRP14 are critical components in the recovery of protein Cys residues from their persulfide derivatives. In addition, we demonstrated that the *NADPH-glutathione reductase-glutaredoxin-GSH* system can also efficiently reduce protein persulfides and inorganic polysulfides. Finally, utilizing ProPerDP, we provided evidence that the *thioredoxin* and *glutathione* systems together orchestrate protein persulfide homeostasis *in vivo*, underlying their pivotal roles in persulfide-mediated signal transduction and protection against oxidative stress.

References:

1. Ida T., Sawa T., Ihara H., Tsuchiya Y., Watanabe Y., Kumagai Y. et al. Reactive cysteine persulfides and S-polythiolation regulate oxidative stress and redox signaling. Proc Natl Acad Sci USA. 2014;111(21):7606-11. PubMed PMID: 24733942.

2. Ono K., Akaike T., Sawa T., Kumagai Y., Wink D.A., Tantillo D.J. et al. Redox chemistry and chemical biology of H2S, hydropersulfides, and derived species: implications of their possible biological activity and utility. Free Radic Biol Med. 2014;77:82-94. PubMed PMID: 25229186.

3. Nagy P. Mechanistic chemical perspective of hydrogen sulfide signaling. Methods Enzymol. 2015;554:3-29. PubMed PMID: 25725513.

4. Greiner R., Palinkas Z., Basell K., Becher D. et al. Polysulfides link H2S to protein thiol oxidation. Antioxid Redox Signal. 2013;19(15):1749-65. PubMed PMID: 23646934.

5. Vasas A., Doka E., Fabian I., Nagy P. Kinetic and thermodynamic studies on the disulfide-bond reducing potential of hydrogen sulfide. Nitric oxide: biology and chemistry / official journal of the Nitric Oxide Society. 2015;46:93-101. PubMed PMID: 25512332.

6. Doka E., Pader I., Biro A., Johansson K., Cheng Q., Ballago K. et al. A novel persulfide detection method reveals protein persulfide- and polysulfide-reducing functions of thioredoxin and glutathione systems. Sci Adv. 2016;2(1):e1500968. PubMed PMID: 26844296.

H2S - Based Anti-inflammatory drugs: lost and found in Translation

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There is a rapidly expanding body of evidence for important roles of hydrogen sulfide in protecting against tissue injury, reducing inflammation, and promoting repair. There is also growing evidence that H_2S can be successfully exploited in drug development. H_2S synthesis and degradation are regulated in circumstances of inflammation and injury so as to promote repair and re-establish homeostasis. Novel H_2S-releasing drugs exhibit enhanced anti-inflammatory and pro-restorative effects, while having reduced adverse effects in many tissues. H_2S is a pleiotropic mediator, having effects on many elements in the inflammatory cascade and promoting the resolution of inflammation and injury. It also contributes significantly to mucosal defence in the gastrointestinal tract, and in host defence against infection. There is strong evidence that novel, H_2S-based therapeutics are safe and effective in animal models, and several are progressing through human trials. A better understanding of the physiological and pathophysiological roles of H_2S continues to be restrained by the lack of simple, reliable methods for measurement of H_2S synthesis, and the paucity of highly selective inhibitors of enzymes that participate in endogenous H_2S synthesis. On the other hand, H_2S donors show promise as therapeutics for several important indications.

Mitochondrial H_2S: a viable therapeutic opportunity?

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Very recently several mitochondrial functions have been shown to be regulated by hydrogen sulfide (H2S), including cellular respiration where H2S is used by mitochondria as an inorganic electron source. Endogenous H2S is produced from mercaptopyruvate by the mitochondrial/cytosolic enzyme 3-mercaptopyruvate sulfurtransferase (3-MST) and intramitochondrial H2S production from 3-MST is crucial for maintaining mitochondrial electron flow and cellular bioenergetics. At least two other enzymatic sources of H2S exist within cells where it is formed from cysteine/homocysteine by cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) located in the cytosol. However, under certain conditions, such as oxidative stress, intracellular levels of H2S and CSE and 3-MST-derived H2S are depleted and genetic or pharmacological inhibition of CSE or 3-MST renders cells/animals more prone to oxidative, inflammation and mitochondrial damage. Several human diseases are associated with oxidative stress, mitochondrial dysfunction and impaired H2S bioavailability, notably diabetes, suggesting improving mitochondrial H2S bioavailability may represent a novel therapeutic strategy for disease treatment. Several slow release donor molecules such as GYY4137 have been shown to protect the vasculature in animals (e.g. hypertension, atherosclerosis, myocardial infarction etc), to inhibit or reverse inflammation (e.g. colitis, arthritis etc) and in isolated cells, protect mitochondria from oxidative injury. However, high concentrations/doses are generally required since H2S generation is not targeted to where it is needed i.e. the mitochondria. With these observations in mind we have designed a series of novel compounds to generate H2S within the mitochondria containing different mitochondrial targeting motifs and H2S donor moieties, notably AP39 and AP123, and novel second generation molecules, and collaborated extensively with many international groups to evaluate the effect of these compounds in vitro and in vivo. We have used fluorescence/confocal microscopy to visualise mitochondrial H2S

generation and tag-switch technology to determine mitochondrial persulfide formation from the donors and their respective control compounds. Under basal conditions in a variety of human and animal cells, mitochondria-targeted H2S donors (0.1-200 nM), but not control compounds, stimulated cellular bioenergetics and ATP production. Under various oxidative stress conditions (e.g. induced by glucose oxidase, peroxide, lipid peroxides, hypochlorite, peroxynitrite, β-amyloid, hyperglycaemia, UV-light etc), mitochondrial damage (e.g. loss of $\Delta\psi m$, oxidant production, mitochondrial DNA and protein damage etc) were inhibited (0.1-300 nM). Mitochondrial protection was also observed with other donors such as GYY4137 but at notably higher concentrations (>200 μM) presumably because H2S generation was not predominantly mitochondrial. With collaborators, we have evaluated the efficacy of some mitochondria-targeted H2S donors, notably AP39, in rat, mouse and large animal models of myocardial and renal ischaemia-reperfusion injury, hypertension, acute and chronic inflammation, neurological injury post-cardiac arrest, UV-light induced skin damage etc. Each study shows mitochondria-targeted donors (but not respective controls) at 'druggable' doses (e.g. 0.7-721 μg/kg) either inhibited or reversed the pathological phenotype in each model. We are currently evaluating the efficacy of AP39, AP123 and second generation compounds in other conditions where mitochondrial dysfunction is a key pathological event as collectively, the above studies strongly suggest that mitochondrial delivery of very low doses of H2S is a viable therapeutic approach to treating human diseases. A snap shot of recent in vivo studies will be presented.

Synthetic H_2S Donors with Defined Release Mechanisms and Tunable Release Rates

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Hydrogen sulfide (H_2S) has emerged as an important small molecule biosynthesized from enzymatic and non-enzymatic pathways, and has joined carbon monoxide (CO) and nitric oxide (NO) as the third endogenously produced gasotransmitter. One key investigative tool in understanding the multifaceted biological roles of H_2S has been the development of synthetic H_2S donors, which release H_2S at controlled release rates akin to enzymatic H_2S synthesis. In addition to providing important research tools, such H_2S donors also have high pharmacological potential for treatment of diseases accompanied by H_2S mis-regulation. Key needs in developing such tools include developing platforms that do not consume thiols during H_2S release and scaffolds that can be triggered to release H_2S on demand and in response to an external stimulus. Motivated by these needs, we have recently focused on developing new chemical strategies for H_2S donation aligned with these requirements. This presentation will focus on newly-developed constructs that provide access to slow-release H_2S donors with tunable release rates as well as donor platforms that can be triggered by either endogenous or biorthogonal triggers.

Hydrogen sulfide and Asthma

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Asthma is a chronic inflammatory disease with hyper-responsive bronchoconstriction and airway remodeling, leading to extensive airway narrowing. The most common type of asthma is allergic asthma. It has been reported that allergic asthma is more common and severe in children than in adults. An epidemiologic study in USA (2001-2009) reported that asthma prevalence is significantly higher in children (<18 years, 9.6%) than in adults (7.7%). Canada has experienced the same higher asthma prevalence in children (4-11 years, ~16%) than in adults (~7%). An European study including 9091 men and women at 29 centers from 14 countries also showed that the incidence of allergic asthma decreases significantly with age.

Human lungs and airways do not fully develop until the 6^{th} to 8^{th} year of life. During the early life, exposure to even mild chemical irritants can have significant effects on lung development. Allergy and allergic triggers have lesser impact on the late-onset asthma. It has been proposed that reduced microbial exposure in early life is the environmental cue for the shifted T_H1/T_H2 balance in the immune system towards the pre-allergic T_H2 response . Whether there is an endogenous cue for the vulnerability to asthma in the early life is unknown.

Our study tested the hypothesis that developmentally regulated cystathionine gamma-lyase expression and H_2S production affect age-dependent vulnerability and severity of allergic asthma. With the advancement of this project, we may develop a new therapeutic avenue by selectively supplementing H_2S to a young population that is vulnerable to allergic asthma attack. (Supported by the Canadian Institutes of Health Research).

Hydrogen sulfide: Antiviral and Anti-Inflammatory Endogenous Gasotransmitter in the Airways. Role in respiratory Syncytial Virus Infection

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Background: Hydrogen sulfide (H_2S) is an endogenous gaseous transmitter whose role in the pathophysiology of several lung diseases has been increasingly appreciated. Our recent studies *in vitro* have shown for the first time that H_2S has an important antiviral and anti-inflammatory activity in respiratory syncytial virus (RSV) infection, the leading cause of bronchiolitis and viral pneumonia in children (1).

Objectives: To evaluate the therapeutic potential of GYY4137, a novel slow-releasing H_2S donor, for prevention and treatment of RSV-induced lung disease, as well as to investigate the role of endogenous H_2S in a mouse model of RSV infection.

Methods: 10-12 week-old BALB/c mice treated with GYY4137, C57BL/6J wildtype control mice (WT), or C57BL/6J mice genetically deficient in the cystathionine γ-lyase enzyme (CSE KO), the major H_2S generating enzyme in the lung, were infected with RSV and assessed for viral replication, clinical disease, airway hyperresponsiveness (AHR) and inflammatory responses. To further determine the effects of exogenous H_2S donor, CSE KO and WT mice were treated with GYY4137 and infected with RSV.

Results: Our results show that intranasal delivery of GYY4137 to RSV-infected BALB/c mice significantly reduced viral replication and markedly improved clinical disease parameters and pulmonary dysfunction compared to vehicle treated controls. The protective effect of H_2S donor was associated with significant reduction of viral-induced proinflammatory mediators and lung cellular infiltrates. Furthermore, CSE $^{-/-}$ mice showed significantly enhanced RSV-

induced lung disease, viral replication and aggravated AHR compared to wild type animals. Administration of exogenous H_2S rescued the RSV-induced disease in CSE KO mice.

Conclusions: Overall our results indicate that H_2S exerts a novel antiviral and anti-inflammatory activity in the context of RSV infection and represents a potential novel pharmacological approach to ameliorate viral-induced lung disease.

References:

1. Li H., Ma Y., Escaffre O., Ivanciuc T., Komaravelli N., Kelley J.P., Coletta C., Szabo C., Rockx B., Garofalo R.P., Casola A. J Virol 2015.,10:5557-68.

Reduction of Highly Oxidizing Heme Redox Intermediates by H_2S and its possible contributions to the Antioxidant Actions of H_2S

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An increasing number of studies reported antioxidant actions of hydrogen sulfide in a variety of biological conditions. Although some Reactive Oxygen Species (ROS) react with sulfide favorably (e.g. the neutrophil oxidant hypochlorous acid oxidize H_2S with a second order rate constant close to the diffusion controlled limit k = 2.3×109 M-1s-1), we argued that due to the low endogenous free sulfide levels compared to protein thiols and glutathione, sulfide is unlikely to exhibit its antioxidant actions via direct scavenging of ROS (1). Instead, we proposed that sulfide's antioxidant properties are related to regulating enzymatic functions (2). Persulfidation-mediated protection of functional and regulatory Cys residues is clearly one of these processes (3).

Here we propose that reduction of highly oxidizing heme redox intermediates is another pathway that contributes to the oxidative stress alleviating actions of H_2S. The neutrophil oxidant myeloperoxidase (MPO) is responsible for generating promiscuous oxidant species (like HOCl, chloramines and free radical species) to kill invading pathogens. However, extracellular release of these oxidants and their unintentional production by circulating MPO was associated with a plethora of inflammatory diseases. Interestingly, in conditions where active MPO was proposed to act as a protagonist (such as reperfusion injury, leukocyte adherence, rheumatoid arthritis, neurodegeneration, and in atherosclerosis), sulfide was reported to be protective. We here present our comprehensive kinetic analyses on the reactions of sulfide with MPO redox intermediate species (4). Our data suggest that reduction of these enzyme forms is a potential biological protective mechanism in MPO-mediated inflammatory conditions.

In addition, here we present data on the favorable reduction of oxidized ferryl hemoglobin (ferryl-Hb) species by sulfide. The measured rate constants of sulfide with ferryl-Hb derivatives represent orders of magnitude faster reactions compared to those of ferryl-Hb with ascorbate and urate. This potentiates that the reaction could contribute to the observed protective actions of H_2S under hemoglobin induced oxidative stress (5). For example, sulfide has beneficial functions in the pathogenesis of atherosclerosis (6) and complication of atherosclerotic lesions where associated with heme-induced atheroma rupture, initiated by erythrocyte hemorrhage (7). We recently obtained evidence that some of sulfide's ateroprotective actions could be related to its intraplaque ferryl-Hb reducing properties (8).

References:

1 Nagy P., Winterbourn C.C. Rapid reaction of hydrogen sulfide with the neutrophil oxidant hypochlorous acid to generate polysulfides. Chem Res Toxicology. 2010;23(10):1541-3. PubMed PMID: 20845929.

2. Nagy P. Mechanistic chemical perspective of hydrogen sulfide signaling. Methods Enzymol. 2015;554:3-29. PubMed PMID: 25725513.

3. Ono K., Akaike T., Sawa T., Kumagai Y., Wink D.A., Tantillo D.J. et al. Redox chemistry and chemical biology of H2S, hydropersulfides, and derived species: implications of their possible biological activity and utility. Free Radic Biol Med. 2014;77:82-94. PubMed PMID: 25229186.

4. Palinkas Z., Furtmuller P.G., Nagy A., Jakopitsch C., Pirker K.F., Magierowski M. et al. Interactions of hydrogen sulfide with myeloperoxidase. Br J Pharmacol. 2015;172:1516-32. PubMed PMID: 24824874.

5. Jeney V., Balla J., Yachie A., Varga Z., Vercellotti G.M., Eaton J.W. et al. Pro-oxidant and cytotoxic effects of circulating heme. Blood. 2002;100(3):879-87. PubMed PMID: 12130498.

6. Yang G., Zhao K., Ju Y., Mani S., Cao Q., Puukila S. et al. Hydrogen sulfide protects against cellular senescence via S-sulfhydration of Keap1 and activation of Nrf2. Antioxid Redox Signal. 2013;18(15):1906-19. PubMed PMID: 23176571.

7. Jeney V., Balla G., Balla J. Red blood cell, hemoglobin and heme in the progression of atherosclerosis. Front Physiol. 2014;5. PubMed PMID: 25324785.

8. Jeney V.L., Potor L., Nagy P., Tolnai E., Vasas A., Balogh E. et al. Elevated Levels Of H2S Inhibit Hemoglobin-Lipid Interactions In Atherosclerotic Lesions. Antioxid Redox Signal. 2016;in revision.

Hydrogen sulfide Regulates *Mycobacterium tuberculosis* Bioenergetics and Progression of Tuberculosis Disease

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Tuberculosis (TB) is a chronic pulmonary disease caused by *Mycobacterium tuberculosis* (*Mtb*) that kills nearly 1.5 million people annually (1). In the majority of infected individuals, *Mtb* persists in a clinically asymptomatic latent state for years and in 5-10% of those infected, it reactivates to cause active TB disease. Recent studies have linked intracellular gases such as carbon monoxide (CO) and nitric oxide (NO) to the induction of TB latency (2,3,4). Hydrogen sulfide (H_2S) shares several biological functions with CO and NO and also plays critical roles in various pathophysiological processes. However, it is unknown whether H2S impacts the outcome of *Mtb* infection and TB disease. We hypothesized that host-derived H_2S influences the intracellular survival of *Mtb* by altering its metabolic and bioenergetic functions. To test this hypothesis, we used high-resolution respirometry to measure bioenergetic functions of *Mtb* following exposure to H_2S and performed infection studies in H_2S-deficient mice. *In vitro* studies revealed that exposure of *Mtb* to H_2S its increases growth, ATP levels and oxygen consumption rate. RNA-seq-based transcriptomic analysis showed that exposure of *Mtb* to low levels of H_2S upregulates several genes associated with dormancy and virulence of *Mtb* during chronic infection. Further investigation showed that H_2S facilitates rapid entry into hypoxia-induced latency and increased survival upon re-aeration, and that H_2S protects *Mtb* against oxidative stress. To determine the relevance of our *in vitro* findings, we infected cystathionine β-synthase (*Cbs*) knockout mice deficient in H_2S production. Compared to wild type control animals, $Cbs^{+/-}$ mice exhibited lower bacillary burden and less lung tissue damage at multiple time points post-infection. Accordingly, the median survival of *Mtb*-infected $Cbs^{+/-}$ mice was significantly increased compared to wild type. Further, immunological analysis revealed reduced Th2 cytokine responses in *Mtb*-infected $Cbs^{+/-}$ mice.

Collectively, our findings establish a novel paradigm whereby a gaseous signaling molecule, H_2S, stimulates *Mtb* proliferation to promote disease. These findings have important implications for understanding how *Mtb* enters, maintains and emerges from a latent infection, and point to CBS as a potential host-directed therapeutic target.

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References:

1. Global Tuberculosis Report WHO (2014).

2. Voskuil M.I. et al. Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program. The Journal of experimental medicine. 2003;198(5):705-713.

3. Kumar A. et al. Heme oxygenase-1-derived carbon monoxide induces the Mycobacterium tuberculosis dormancy regulon. Journal of Biological Chemistry. 2008;283(26):18032-18039.

4. Shiloh M.U. et al. Mycobacterium tuberculosis senses host-derived carbon monoxide during macrophage infection. Cell Host Microbe. 2008;3(5):323-330.

Hydrogen sulfide as new player of Amyotrophic Lateral Sclerosis

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By introducing hydrogen sulphide (H_2S) as an endogenously generated neuromodulator, a large body of data about the role of H_2S has been accumulated. To date contrasting hypotheses emerge about its function in the nervous system. Our recent data showed poisonous levels of H_2S in cerebrospinal fluid of patients with Amyotrophic Lateral Sclerosis (ALS) and in the familial ALS mouse model $SOD1^{G93A}$ (1). ALS is a lethal disease characterized by a progressive motor neuron degeneration. Many etiologic factors are implicated, such as glial inflammation, excitotoxicity and toxic accumulation by misfolded proteins. However, it is accepted that a severe mitochondrial dysfunction leads to an unavoidable neuronal death. H_2S is mainly produced in the brain by astrocytes and microglia through the cystathionine-β-synthase (CBS), a cytoplasmatic enzyme that accumulates in mitochondria under oxygen sensitive conditions. H_2S inhibits complex IV of the mitochondrial respiratory chain; on the other side impairments in the complex IV-drive respiration have been described in $SOD1^{G93A}$ mice.

Therefore, the aim of this study is to further unravel the complexity of H_2S metabolism and the molecular mechanisms through which H_2S could contribute to the ALS-related neurodegeneration.

We developed specific HPLC test to measure H_2S levels in tissues and in spinal cord cultures of $SOD1^{G93A}$ mice. In order to highlight the key pathways and the putative protein-protein interactions, deeper untargeted and targeted proteomics analysis were performed on neuronal tissues derived from $SOD1^{G93A}$ mice at different developmental/disease stages. Differential protein expression of total protein extracts and mitochondrial enriched fractions were evaluated.
We looked putative dysregulated biological processes linked to H_2S metabolism between the cytosolic and mitochondrial compartments.

We focused on key proteins that are directly affected by H_2S and that could be relevant for ALS such as GADPH and actin; on the other side, we focused on ALS proteins with reactive cysteine that may be regulated by H_2S such as mitochondrial complex subunits and SOD1.
Our data show that the increased H_2S amount in ALS could further distress an already compromised mitochondrial function. H_2S toxic effects seem to associate with phenotype development in ALS. Our study introduces H_2S as a new player to the cohort of pro-inflammatory/degenerative factors that could be involved in the etiology of ALS.

References:

1. Davoli A., Greco V., Spalloni A. et al. Evidence of Hydrogen Sulphide involvement in Amyotrophic Lateral Sclerosis. Ann Neurol. 2015 Jan 27. doi:10.1002/ana.24372.

Dysregulated ATF4 regulation and imbalanced Redox Homeostasis caused by Cysteine Stress mediates Neurodegeneration in Huntington's disease

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Huntington's disease is a neurodegenerative disorder characterized by expansion of polyglutamine repeats in the protein huntingtin. Mutant huntingtin causes widespread damage leading to elevated oxidative stress, motor and cognitive dysfunction. We have previously shown that cystathionine gamma lyase (CSE), the biosynthetic enzyme for cysteine, is depleted in HD and mediates disease progression by altering redox homeostasis. Cysteine, being a component of the endogenous antioxidant glutathione and the precursor of the gaseous signaling molecule hydrogen sulfide plays a central role in mitigating redox imbalance in cells. Sequestration of SP1, the transcription factor for basal CSE expression, by mutant huntingtin is responsible for this decline. Here, we report that activating transcription factor 4 (ATF4), the master regulator of amino acid biosynthetic enzymes including CSE under nutrient stress is dysfunctional in Huntington's disease.

This abnormality results from chronic oxidative stress caused by "cysteine stress" as result of CSE depletion. Mitigating oxidative imbalance by antioxidants rescues the protective response mechanisms to stress. Our findings reveal the molecular basis for the decline in protective pathways during neurodegeneration in Huntington's disease.

References:

1. Paul BD, Sbodio JI, Xu R, Vandiver MS, Cha JY, Snowman AM, Snyder SH. Cystathionine γ-lyase deficiency mediates neurodegeneration in Huntington's disease. Nature. 2014 May 1;509(7498):96-100.

2. Paul B.D., Snyder S.H. Neurodegeneration in Huntington's disease involves loss of cystathionine γ-lyase. Cell Cycle. 2014;13(16):2491-2493.

3. Paul B.D., Snyder S.H. H2S: A Novel Gasotransmitter that Signals by Sulfhydration. Trends Biochem Sci. 2015 Nov;40(11):687-700.

Endogenous sulfide Regulation of Arterial Vascular Remodeling

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Arterial vascular remodeling occurs in response to different stimuli in order to maintain vascular tone and blood flow. Exogenous hydrogen sulfide has been reported to modulate vascular growth and remodeling under several conditions. However, the importance of endogenous sulfide regulation of arterial vascular remodeling remains poorly understood. Our group examined arterial vascular changes in two different experimental models with increased or decreased blood flow in wild type and CSE knockout mice. Initial studies were performed using the femoral artery ligation (FAL) model, whereby blood flow is increased across gracillis collateral arteries. CSE genetic deficiency significantly blunted FAL mediated arteriogenesis of gracillis arteries resulting in abnormal restoration of hind limb reperfusion over time. Deficiency of CSE blunted FAL mediated monocyte/macrophage recruitment, which is a key cellular mediator of arteriogenesis activity. Importantly, diallyl trisulfide (DATS) therapy rescued FAL dependent arteriogenesis and monocyte recruitment in CSE deficient mice. Arterial remodeling changes were next examined in the partial carotid ligation model resulting in low oscillatory blood flow. Partial carotid ligation of the left carotid artery reduced blood flow similarly in both wild type and CSE knockout mice. However, medial thickening and luminal narrowing occurred normally in wild type but was blunted in CSE deficient mice. Importantly, monocyte infiltration of the left carotid artery was completely blunted in CSE deficient compared to wild type mice. These changes were associated with decreased endothelial cell inflammatory activation and augmented carotid vessel nitric oxide metabolite levels. Together, these findings reveal a complex and critical role of endogenous sulfide metabolism and CSE expression in regulating monocyte/macrophage dependent arterial remodeling.

Nutrient Restriction Triggers Angiogenesis via regulation of Endogenous H_2S Production

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Angiogenesis, the formation of new blood vessels by endothelial cells (ECs), is an adaptive response to tissue ischemia orchestrated by vascular endothelial growth factor (VEGF) upon vessel occlusion, exercise or tumor growth. Hypoxia is the best-understood trigger of VEGF expression via the transcription factor HIF1alpha. Nutrient deprivation is inseparable from hypoxia upon ischemia, yet its role in angiogenesis is poorly characterized. Here, we report that dietary sulfur amino acid restriction in mice promoted new capillary growth in skeletal muscle independent of hypoxia or HIF1alpha, but instead requiring the amino acid-sensing eIF2alpha kinase GCN2 and the transcription factor ATF4. GCN2/ATF4 activation increased cystathionine-gamma-lyase expression and pro-angiogenic hydrogen sulfide (H_2S) production. Amino acid restriction is thus a powerful trigger of angiogenesis independent of hypoxia.

Protection against Ischemia/reperfusion Injury by H_2S donors: the Role of Mitochondria

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Hydrogen sulfide (H_2S) is a new signaling molecule with important effects in the cardiovascular system. In the blood vessels, H_2S reduces smooth muscle tone and promotes angiogenesis. In the heart, H_2S exerts cardioprotective effects by reducing apoptosis, limiting oxidative stress and preventing structural changes that develop with heart failure. To harness the therapeutic potential of H_2S, a number of H_2S donors have been developed; these agents differ in the rate of H_2S release, as well as in the sub-cellular compartment in which H_2S is released. We have previously shown that H_2S salts (NaHS and Na_2S), GYY4137 (a slowly releasing agent), thiovaline (a donor with intermediate H_2S releasing rate) and AP39 (a mitochondrial-targeted H_2S donor) all reduce infarct after LAD ligation to approximately the same extent. We observed that the effects of Na_2S, but not those of AP39, are associated with enhanced eNOS-phosphorylation on Ser1176.

In line with these observations, herein we report that administration of the endothelial nitric oxide synthase (eNOS) inhibitor N-nitro-L-arginine-methyl-ester (L-NAME) reversed the infarct-limiting effects of Na_2S in ischemia-reperfusion (I/R) injury (infarct/risk area 17.8$\pm$ 1.8% for Na_2S vs 32.9$\pm$ 2.4% for LNAME+Na_2S), while the cardioprotective effects of GYY-4137, thiovaline and AP39 were not affected by NOS inhibition. Treatment of animals with this Na_2S lead to enhanced vasodilator-stimulated phosphoprotein (VASP) phosphorylation, a marker of cGMP-dependent protein kinase (PKG) activation. The protective effect of Na_2S was limited by the PKG inhibitor DT-2 (29.9 $\pm$ 1.8% for DT-2 + Na_2S). In contrast, we did not observe VASP phosphorylation on Ser239 after AP39, nor was the infarct-limiting effect of AP39 affected by PKG inhibition (16.5$\pm$ 2.3% vs 14.4$\pm$ 3.7% for AP39 and DT-2 + AP39).

Opening of the mitochondrial permeability transition pore (mPTP) is considered to be a major cause of cell death in ischemia-reperfusion injury of the heart. Therefore, we tested the effects of Na_2S and AP39 in mice lacking cyclophilin-D (CyD), a key regulator of mPTP. CyD knockout mice exhibited smaller infarcts after LAD ligation compared to wild-type mice (39.2$\pm$1.8% vs 17.7$\pm$2.2% for wild-type and CyD KO, respectively). Administration of both Na_2S and AP39 further reduced infarct size in CyD KO animals (8.9$\pm$3.1% vs 10.4$\pm$3.6% for Na_2S and AP39), suggesting that the cardioprotection they confer is CyD-independent. To test if H_2S produced from Na_2S, GYY4137 and AP39 exerts direct effects on mitochondria, we evaluated the Ca^{2+} retention capacity of isolated heart mitochondria following a series of Ca^{2+} pulses in the absence of presence of cyclosporine A (CsA) and increasing H_2S donors concentrations. In these experiments we observed that Na_2S (40nM-40μM) and GYY4137 (10-300μM) did not alter Ca^{2+} uptake by mitochondria, while AP39 (300nM) significantly increased the mitochondrial Ca^{2+} retention capacity, both in the absence and in the presence of CsA.

We conclude that Na_2S limits infarct size in a NO/cGMP/PKG-dependent pathway; Na_2S does not appear to have a direct affect on mitochondrial function. In contrast, the NO-independent cardioprotection exhibited by AP39 could result from a direct inhibitory effect on mPTP opening, acting at a site different than CyD.

Hydrogen sulfide Mitigates Myocardial Infarction via Promotion of Mitochondrial Biogenesis-Dependent M2 Polarization of Macrophages

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Macrophages are of key importance for tissue repair after myocardial infarction (MI) (1, 4), activation (polarization) of macrophages results in the generation of classically activated (M1) and alternatively activated (M2) subsets which exhibit distinct cell marker expression and diverse immunological functions (5). Hydrogen sulfide (H2S) has been shown to exert cardioprotective effects in MI (2, 3). However, the mechanisms by which H_2S modulates cardiac remodeling and repair post-MI remain to be clarified. In our current study, we show H_2S supplementation ameliorated pathological remodeling and dysfunction post-MI in WT and CSE-KO mice, resulting in decreased infarct size and mortality, accompanied by an increase in the number of M2-polarized macrophages at the early stage of MI. Strikingly, adoptive transfer of NaSH-treated BMMs into WT and CSE-KO mice with depleted macrophages also ameliorated MI-induced cardiac functional deterioration. Further mechanistic studies demonstrated that NaSH-induced M2 polarization was achieved by enhanced mitochondrial biogenesis and fatty acid oxidation (FAO). Our study shows, for the first time, that H_2S may have the potential as a therapeutic agent for MI *via* promotion of M2 macrophage polarization.

References:

1. Frantz S. et al. Monocytes/macrophages prevent healing defects and left ventricular thrombus formation after myocardial infarction. FASEB. 2013 J. 27: 871-881.

2. Pan L.L., Liu X.H., Gong Q.H., Yang H.B. & Zhu Y.Z. Role of cystathionine γ-lyase/hydrogen sulfide pathway in

cardiovascular disease: a novel therapeutic strategy?. Antioxid Redox Signal. 2012;17:106-118.

3. Calvert J.W., Coetzee W.A., Lefer D.J. Novel insights into hydrogen sulfide–mediated cytoprotection. Antioxid Redox Signal. 2010;12:1203-1217.

4. Van Amerongen M.J., Harmsen M.C., van Rooijen N., Petersen A.H., & van Luyn M.J. Macrophage depletion impairs wound healing and increases left ventricular remodeling after myocardial injury in mice. Am J Pathol. 2007;170:818-829.

5. Lambert J.M., Lopez, E.F. & Lindsey, M.L. Macrophage roles following myocardial infarction. Int. J. Cardiol. 2008;130:147-158.

Molecular Mechanism of H_2S-Induced Cardioprotection

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Hydrogen sulfide (H_2S) plays roles in cardiovascular homeostasis and disease. Several studies have investigated a protective effect of H_2S against myocardial ischaemia-reperfusion injury, but no clear mechanism has been identified. Part of the inconsistency in experimental findings is related to the use of unstable inorganic sulfide salts to generate H_2S but these may trigger off-target effects. Moreover, H_2S was delivered in dosing regimens which are clinically irrelevant. Taken together, the molecular mechanism by which H_2S induces cardioprotection and the cellular compartments involved still need further investigation in clinically relevant models and regimens. In the present study, we investigated the molecular targets of two H_2S donors (GYY4137, a slow-releasing H_2S donor, and AP39(1), a mitochondria-targeting H_2S donor) in an in vivo model of myocardial ischaemia-reperfusion injury. Thiobutabarbital-anaesthetised male rats were subjected to 30 minutes of coronary artery occlusion followed by reperfusion for 120 minutes. Infarct size measured by tetrazolium staining is reported as a percentage of area at risk. Administration of both H_2S donors (226 µmol/kg for GYY4137 and 1µmol/kg for AP39) significantly limited infarct size when given 10 minutes before reperfusion. Neither compound had any significant effect on haemodynamics. Decomposed GYY4137, (synthesized as described) (2) had no effect on either infarct size or haemodynamics. Similarly, equal doses of AP39 controls consisting of the mitochondria-targeting moiety (AP219)[3] or the H_2S releasing moiety (ADT-OH) (3)exerted any infarct limitation. Concomitant administration of the constitutive nitric oxide synthase (eNOS) inhibitor N-nitro-L-arginine-methyl-ester (L-NAME) with GYY4137 attenuated its cardioprotection, but did not abrogate it. The PI3K inhibitor (LY294002) completely abolished GYY4137-induced cardioprotection. Interestingly, AP39-induced cardioprotection was not abrogated by either L-NAME, LY294002 or selective guanylyl cyclase inhibitor ODQ.

Western blot analysis of myocardium harvested in early reperfusion showed that GYY4137 increased Ser^{473}Akt phosphorylation. There was also partial dependency of GYY4137 cardioprotection on NO availability as Ser^{1177}eNOS phosphorylation was increased by GYY4137. This increase was abolished by both L-NAME and LY294002. GYY4137 also increased Ser^{9}GSK phosphorylation inhibiting its activity at early reperfusion. However, GSK phosphorylation was abrogated by LY294002 or L-NAME. There was no significant difference in ERK1/2 phosphorylation in GYY4137 treated heart at early reperfusion. In sharp contrast, treatment with AP39 had no significant effect on either Akt, eNOS, GSK-3β or ERK1/2 activation/activity at early reperfusion.

These data suggest that slow release of H_2S at early reperfusion protects that heart against myocardial reperfusion injury resulting in infarct limitation. GYY4137 activates PI3K/Akt/eNOS/NO-dependent signaling pathway at reperfusion and inhibits the activity of GSK-3β. Accordingly, it seems plausible that GYY4137 attenuates the susceptibility of mitochondrial permeability transition pore opening at early reperfusion. We also saw that in contrast to GYY4137, the infarct limiting effect of AP39 at reperfusion is independent of cytosolic signaling e.g. without detectable PI3K/Akt/eNOS/NO involvement, further supporting the notion that it directly targets the mitochondria (2-4). We are currently further characterization the cardioprotective mechanism(s) of GYY4137 and AP39 using different subpopulations of myocardial mitochondria, namely, subsarcolemmal and interfibrillar mitochondria.

References:

1. Alexander et al. MedChemCommun. 2015;6:1649-55.

2. Szczesny et al. Nitric Oxide 2014;41:120-130.

3. Tomasova et al. Nitric Oxide 2015;46:131-44.

4. Ikeda et al. Nitric Oxide 2015;49:90-6.

Hydrogen sulfide and Cancer: pathomechanisms and therapeutic opportunities

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In various forms of cancer (including colorectal and ovarian cancer), increased production of hydrogen sulfide (H_2S) from cystathionine-β-synthase (CBS) plays an important role in promoting cellular bioenergetics, proliferation and migration. Pharmacological inhibition or genetic silencing of CBS exerts antitumor effects *in vitro* and *in vivo*, and potentiates the efficacy of current standard-of-care anticancer therapeutics. In the current presentation, recently published studies will be overviewed documenting a critical role for CBS-dependent H_2S production in cancer cell proliferation and tumorigenesis, and molecular mechanisms will be presented by which H_2S provides a pro-tumor-growth environment. Next, the state-of-the-art of CBS inhibition will be discussed, including the complex pharmacology of aminooxyacetic acid, which includes the inhibition of CBS, as well as the inhibition of several other PLP (pyridoxal phosphate) dependent enzymes. Finally, a novel pharmaceutical strategy will be presented, which enhances the cellular uptake, the cell-based antiproliferative efficacy and the in vivo antitumor potency of AOAA via the prodrug approach.

Involvement of Hydrogen sulfide Pathway in Human Melanoma Development and Progression

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Melanoma is the most common form of skin cancer. Given its high mortality, the interest in the search of preventive measures is growing significantly. Hydrogen sulfide (H_2S) is a gaseous signaling molecule that plays important roles in a variety of biological functions in health and disease (Paul & Snyder, 2015). Emerging data indicate that H_2S is also involved in the regulation of tumor cell biology (Hellmich MR, Szabo C. 2015). However, the effects of H_2S on cancer are controversial and still unclear. It has been shown that H_2S induces DNA damage and alter cell cycle in various mammalian cells. Endogenously produced or exogenously released H_2S has a role in the accumulation or proliferation of cells and further may provide the fundamentals for development of a novel therapeutic approach in conditions associated with uncontrolled cell growth (Baskar and Bian, 2011). However, the potential biological and clinical significance of H_2S are subject of intense debate in recent years and despite considerable progress in our understanding about H_2S, much still needs to be learned about their production at the site of tissue injury and its downstream signaling pathways on cell growth. It is also important to emphasize that in the recent literature different roles have been attributed in several types of cancer to CBS-derived H_2S and to CSE-derived H_2S. Moreover many of the biological responses to H_2S follow a biphasic dose-response: while low concentrations of H_2S are responsible of physiological and cytoprotective effects, high concentrations result cytotoxic (Szabo, 2016). We have recently demonstrated that the metabolic pathway l-cysteine/CSE/H_2S is involved in human melanoma progression and that natural and synthetic H_2S releasing agents display anti-tumoral effect (Panza et al., 2015). Our results establish H_2S-donors as new potential agents in the treatment of human metastatic melanoma and represent a very promising strategy to improve the fight against cancer.

Inflammatory bowel diseases (IBDs) are considered major risk factors for colorectal cancer (Eaden et al., 2001). Several evidences link H_2S to colonic nociception, IBD and colorectal cancer. The exact mechanisms and pathways by which H_2S exerts its multitude of effects are not yet fully understood, but its involvement in physiological and pathophysiological conditions of the colon is becoming evident and several studies support the anti-inflammatory activity of H_2S in experimental colitis (Xu et al., 2005; Fiorucci et al., 2007; Wallace et al., 2009). We have also investigate on the role of H_2S during the pathogenesis of colitis-associated cancer induced by infection with the intestinal bacteria *Helicobacter hepaticus* (H*h*). The results of our study demonstrate that both enzymes were constitutively expressed in the colon of healthy mice and that CBS, but not CSE, was significantly reduced during colitis development.

In conclusion, our findings point towards a protective role of H_2S in inflammatory based model of cancer and identify a new promising strategy to improve the fight against this terrible disease.

Targeting the Bile Acid activated Receptors Gaseous - transmitter axes in the Splanchnic and Systemic Circulation in the Treatment of Liver and Metabolic Disorders

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Cystationine –γ-liase (CSE) is a key regulatory enzyme in the biosynthetic pathway that lead to generation of hydrogen sulfide (H_2S). CSE is highly expressed in the portal and systemic circulation suggesting a role for H_2S in the regulation of both portal and systemic circulation. Nevertheless, molecular mechanisms that regulate CSE activity are still poorly defined. Bile acids activated receptors are a family of nuclear (FXR, PXR, LXR and VDR) and G protein coupled receptors (GPBAR1) highly expressed in entero-hepatic tissues and vascular tissues that are activated by bile acids. Primary and secondary bile acids circulate in portal flow and systemic circulation providing a mechanism of regulation for CSE. A number of evidence support the notion that both FXR and GPBAR1 are involved in the regulation of gaseous vasodilators in the portal and systemic circulation, providing a connecting food ingestion, intestinal microbiota and host metabolism to the vascular system. This regulation appears to be altered in liver diseases contributing to portal hypertension and systemic vasodilation observed in cirrhosis. Additionally, bile acids increase their systemic concentration after feeding, suggesting a putative role for these mediators in post-prandial splanchnic and systemic vasodilation.

The review will also include data from clinical studies demonstrating that administration of FXR and GPBAR1 ligands might affect mean arterial pressure in patients with liver steatosis and metabolic syndrome.

Vascular Effects of p-Carboxyphenyl-Isothiocyanate, a novel H_2S-donor

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Hydrogen sulfide (H_2S), is a pivotal mediator in cardiovascular physiology. This gasotransmitter evokes vasorelaxing effects through different mechanisms of action, such as the inhibition of phosphodiesterases and activation of vascular KATP and Kv7 potassium channels. Indeed, impaired production of H_2S contributes to the pathogenesis of important cardiovascular disorders (1). Therefore, exogenous compounds, acting as H_2S-releasing agents, are viewed as promising therapeutic agents for cardiovascular diseases. This work aimed at evaluating the H_2S-releasing properties of the p-Carboxyphenyl-Isothiocyanate (PhNCS-COOH) derivative and its vascular effects.

H_2S release was first determined by the amperometric approach and unequivocally confirmed by gas chromatography/mass spectrometry. Unlike NaHS, a fast H_2S-donor widely used in the laboratory but unsuitable for clinical use, PhNCS-COOH exhibited a slow H_2S-releasing profile, similar to the slow-releasing reference drugs diallyldisulfide (DADS) and GYY4137. H_2S release from PhNCS-COOH occurred only in the presence of an excess of L-Cysteine: this thiol-dependency has been viewed as a particularly advantageous property, because it allows this compound to release H_2S only in a biological environment. The vascular activity of PhNCS-COOH was tested in rat aorta and coronary arteries. Like NaHS, PhNCS-COOH displayed concentration-dependent vasorelaxing effects on endothelium-denuded rat aortic rings. These effects were significantly antagonized by the Kv7 blocker XE991. PhNCS-COOH also inhibited the vasoconstricting effect of noradrenaline (NA), with greater potency than NaHS.

In addition, the isothiocyanate derivative increased basal coronary flow similarly to NaHS. Furthermore PhNCS-COOH was more

effective than NaHS in counteracting the coronary vasoconstriction induced by angiotensin II.

Since H_2S is known to hyperpolarize vascular smooth muscle by activating K_{ATP} and Kv7 channels (1,2), we evaluated its effects on the membrane potential of human aortic smooth muscle cells (HASMC) using a membrane potential sensitive fluorescent dye. Like the reference Kv7 activator Retigabine, PhNCS-COOH evoked a marked hyperpolarization, largely due to the activation of Kv7 channels (3).

In conclusion, PhNCS-COOH can be viewed as a new suitable slow H_2S-releasing drug, endowed with vasorelaxing effects, typical of the endogenous gasotransmitter. PhNCS-COOH might be employed as a novel chemical tool in basic studies and in the development of original drugs in cardiovascular diseases.

References:

1. Martelli A., Testai L., Breschi M.C., Blandizzi C., Virdis A., Taddei S., Calderone V. Hydrogen sulphide: novel opportunity for drug discovery Med Res Rev. 2012. Nov;32(6):1093-130.

2. Martelli A., Testai L., Breschi M.C., Lawson K., McKay N.G., Miceli F., Taglialatela M., Calderone V. Vasorelaxation by hydrogen sulphide involves activation of Kv7 potassium channels. Pharmacol Res. 2013 Apr;70(1):27-34.

3. Martelli A., Testai L., Citi V., Marino A., Bellagambi F.G., Ghimenti S., Breschi M.C., Calderone V. Pharmacological characterization of the vascular effects of aryl isothiocyanates: is hydrogen sulfide the real player? Vascul Pharmacol. 2014 Jan;60(1):32-41.

The activity of the sulfide oxidation pathway fine-tunes cellular affinity for oxygen

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Sulfide is oxidized by a sulfide quinone reductase (SQR) and is a genuine mitochondrial substrate, which yields electrons to respiratory chain arriving ultimately at cytochrome oxidase where they combine with oxygen to generate water (1). In addition, sulfide is also a strong inhibitor of this cytochrome oxidase, similar to the other gaseous transmitters NO, CO or to cyanide. Low concentrations of sulfide are quickly eliminated by SQR (2). In contrast, when sulfide concentration increases and reaches the low micromolar range mitochondrial respiration is inhibited (3).
Endogenous sulfide releasing rates have different origins (transsulfuration pathway, cysteine metabolism, reductive sulfur metabolism of the gut microbiota). Hence the final concentration of sulfide reflects the balance between sulfide releasing and sulfide consuming reactions. When compared to other metabolic fluxes sulfide release/oxidation appears of modest intensity. However, if sulfide release is unchecked the inhibiting concentration may appear in tissues within few dozens of seconds. SQR and mitochondrial respiration constitute the major sulfide sink. Consequently, sulfide inhibits its own elimination. This is the basis of a positive feedback loop that could accelerate greatly the establishment of respiratory inhibition when sulfide release exceeds elimination (oxidation) rate.
In addition to its dependence on sulfide concentration the sulfide oxidation pathway is dependent on oxygen. Consequently, we aimed to evaluate if and how sulfide release and oxidation impacts on the cellular affinity for oxygen. In agreement with a positive feedback mechanism when sulfide delivery approaches the maximal sulfide oxidation rate cells become exquisitely dependent on oxygen availability. The balance between sulfide-releasing and sulfide-oxidizing rates is the relevant parameter rather than the absolute values of these rates.

Conclusions: i) within the context of continuous release of sulfide stemming from cellular metabolism, alterations in the activity of the sulfide oxidation pathway fine-tunes the cell's affinity for oxygen, and; *ii)* a decrease in the expression of the sulfide oxidation pathway (SQR) greatly enhances the cell's dependence on oxygen concentration. The relevance of this mechanism both for oxygen detection and for the control of oxygen usage/diffusion within tissues should now be considered.

References:

1. Goubern M., Andriamihaja M., Nübel T., Blachier F., Bouillaud F. Sulfide, the first inorganic substrate for human cells. FASEB J. 2007 Jun;21(8):1699-706.

2. Szabo C., Ransy C., Módis K., Andriamihaja M., Murghes B., Coletta C., Olah G., Yanagi K., Bouillaud F. Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part I. Biochemical and physiological mechanisms. Br J Pharmacol. 2014 Apr;171(8):2099-122.

3. Abou-Hamdan A, Guedouari-Bounihi H, Lenoir V, Andriamihaja M, Blachier F, Bouillaud F. Oxidation of H2S in mammalian cells and mitochondria. Methods Enzymol. 2015;554:201-28.

Hydrogen sulfide inhibits RANGE Toxicity through reducing its dimer formation

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Advanced glycation end products (AGEs) are important factors for developing or worsening many degenerative human diseases. We previously reported that hydrogen sulfide (H_2S) reduces the formation of AGEs and their subsequent harmful effects in brain cells (1). The present study was designed to investigate the effect of H_2S on stability of the receptor for AGE (RAGE). We found that NaHS (10-100 μM, 30 min) produced protective effects against H_2O_2 or $A\beta_{1-42}$-induced cytotoxicity in SHSY-5Y cells expressing RAGE. Treatment with NaHS also significantly reduced $A\beta_{1-42}$–induced cellular senescence. Western blotting analysis showed that treatment with either H_2O_2 or $A\beta_{1-42}$ upregulated expression of RAGE, and this effect was significantly attenuated by either pretreatment with NaHS or over-expression of cystathionine β-synthase (CBS), an enzyme for endogenously producing H_2S in brain cells. Moreover, NaHS also abolished H_2O_2-enhanced RAGE dimerization in transfected HEK293 cells. To confirm this effect, we employed a split GFP complementation strategy that enables direct observation of protein interactions in the ER. We found that H_2S reduced RAGE expression in the plasma membrane. The cysteine residues (C259 and C310) in the C2 domain of RAGE are responsible for forming the intermolecular disulfide bonds (2). To study the mechanism underlying the effect of H_2S on RAGE trafficking, the two cysteine residues were mutated to serine (C259S/C310S, DM-RAGE). Treatment with H_2O_2 also increased the expression of DM-RAGE in HEK293 cells. However, both endogenous and exogenous application of H_2S failed to reverse this effect. Immunofluorescence analysis

demonstrated that H_2O_2 upregulated the expression of WT-RAGE on cell membrane. Treatment with NaHS attenuated the effects of H_2O_2 on WT-RAGE. Moreover, NaHS at 10-100 µM induced S-sulfhydration on WT-RAGE in a dose-dependent manner, and this effect was absent in DM-RAGE. These data suggest that NaHS may induce S-sulfhydration of RAGE at C259/C310 residues and therefore prevent formation of the double-disulfide-linked dimeric structure of RAGE. Since RAGE dimerization in the ER is critical for its biogenesis, we studied the effect of H_2S on the stability of RAGE. Cycloheximide chase and ubiquitination assays showed that the half life of DM-RAGE was markedly shorter than that of WT-RAGE. NaHS reduced the half-life of WT-RAGE but not that of DM-RAGE. Taken together, our data suggest that H_2S reduces the formation of RAGE dimer and impairs its stability. The lowered membrane abundance of RAGE therefore helps to protect cells against various RAGE mediated pathological effects.

References:

1. Liu Y.Y., Nagpure B.V., Wong P.T., Bian J.S. Hydrogen sulfide protects SH-SY5Y neuronal cells against d-galactose induced cell injury by suppression of advanced glycation end products formation and oxidative stress. Neurochem Int . 2013 62: 603-609.

2. Wei W., Lampe L., Park S., Vangara B.S., Waldo G.S., Cabantous S., Subaran S.S., Yang D., Lakatta E.G., Lin L. Disulfide bonds within the C2 domain of RAGE play key roles in its dimerization and biogenesis. PLoS One 2012 7, e50736.

3. Xie J., Mendez J.D., Mendez-Valenzuela V., Aguilar-Hernandez M.M. Cellular signalling of the receptor for advanced glycation end products (RAGE). Cell Signal. 2013 25: 2185-2197.

Inhibition of H_2S Biosynthesis Sensitizes Lung Adenocarcinoma Cells to Genotoxic Agents

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Increasing experimental evidence supports the manipulation of endogenous hydrogen sulfide (H_2S) levels as a novel therapeutic approach for treating a variety of solid tumors. We have reported that inhibition or depletion of H_2S-producting enzyme, cystathionine beta-synthase (CBS), reduces the proliferation of colon cancer cells in culture and tumor growth in vivo (1). The importance CBS in ovarian cancer (2) and the role of another H_2S-generating enzyme, cystathionine gamma lyase (CSE) in melanomas (3) has also been reported. However, the molecular mechanisms underlying the tumorpromoting effects of this gaseous transmitter are only beginning to be elucidated. Here we report, for a first time, that human lung adenocarcinomas aberrantly express higher levels of CBS and CSE as well as a third H_2S-generating enzyme, 3-mercaptopyruvate sulfurtransferase (3-MST), than patient-matched normal lung tissue adjacent to the tumor.

Similarly, overexpression of these enzymes was detected in the human lung adenocarcinoma-derived cell lines (A549 and H1944), but not in the normal lung epithelial cell line, BEAS2B. Using both inhibitors of H_2S biosynthesis and selective gene silencing, we showed that collectively all three enzymes contribute to increased H_2S production by the lung cancer cells and tumor tissue, when compared to the normal controls. We found that enhanced H_2S production by A549 and H1944 cells regulates mitochondrial (mt) DNA repair, but not nuclear DNA repair. Importantly, the increased endogenous H_2S production by the lung cancer cells has beneficial effect cellular bioenergetics, viability, migration, invasiveness and survival. Conversely, pharmacological inhibition of the H_2S-generating enzymes sensitizes lung cancer cells to chemotherapeutic drugs via induction of

mitochondrial dysfunction, which, in turn, triggers cancer cell death in culture and reduced human tumor xenograft growth in immune-compromised mice. Lung cancer is the leading cause of cancer deaths worldwide.

The data presented here support the development of novel therapeutic strategies targeting H_2S biosynthesis for the treatment of patient with advance lung adenocarcinoma.

References:

1. Szabo C., Coletta C., Chao C., Módis K., Szczesny B., Papapetropoulos A., Hellmich M.R. Tumor-derived hydrogen sulfide, produced by cystathionine-β-synthase, stimulates bioenergetics, cell proliferation, and angiogenesis in colon cancer. Proc Natl Acad Sci U S A. 2013;110:12474-9.

2. Bhattacharyya S., Saha S., Giri K., Lanza I.R., Nair K.S., Jennings N.B., Rodriguez Aguayo C., Lopez-Berestein G., Basal E., Weaver A.L., Visscher D.W., Cliby W., Sood A.K.,Bhattacharya R., Mukherjee P. Cystathionine beta-synthase (CBS) contributes to advanced ovarian cancer progression and drug resistance. PLoS One. 2013;8: e 79167.

3. Panza E., De Cicco P., Armogida C., Scognamiglio G., Gigantino V., Botti G., Germano D., Napolitano M., Papapetropoulos A., Bucci M., Cirino G., Ianaro A. Role of the cystathionine γ lyase/hydrogen sulfide pathway in human melanoma progression. Pigment Cell Melanoma Res. 2015;28:61-72.

Hydrogen sulfide Attenuates Cisplatin-induced Nephrotoxicity without Compromising its Anti-cancer Benefit

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Cisplatin is a major therapeutic drug to treat solid tumors. Unfortunately, the clinical usage is limited by its severe adverse effects like nephrotoxicity. The mechanisms of cisplatin-induced nephrotoxicity involve activation of mitogen-activated protein kinase (MAPK) related mitochondrial apoptosis and endoplasmic reticulum (ER) stress. This study was designed to investigate whether hydrogen sulfide (H_2S, an endogenous mediator) can prevent cisplatin-induced nephrotoxicity and to study the underlying mechanism. We found in the present study that NaHS, an H_2S donor, largely attenuated cisplatin-induced cell injury in renal proximal tubule (RPT) cells. NaHS reversed cisplatin-induced cleavage of caspase 9 and caspase 3. This effect implies that H_2S may suppress cisplatin-mediated mitochondrial apoptotic pathway. In addition, NaHS also inhibited the activation of caspase 12, suggesting the alleviation of ER stress. Our study also demonstrated that the beneficial effects of H_2S were mediated by the suppression of cisplatin-induced MAPK activation in RPT cells. Interestingly, we did not observe a similar effect of H_2S in several cancer cell lines. In conclusion, our data demonstrated for the first time that H_2S may alleviate cisplatin-induced nephrotoxicity without affecting its chemotherapeutic benefits. Suppression of both mitochondrial apoptosis and ER stress in RPT cells may contribute to its protective effects against nephrotoxicity. Thus, H_2S may have the potential value to reduce the adverse effects of chemotherapy in cancer.

Sulfide-resistant Bacterial Respiration: a New Role for Cytochrome bd Oxidase

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Many prokaryotic species code for orthologs of the mammalian H_2S-synthesizing enzymes and generate H_2S through amino acid metabolism and dissimilatory sulfate reduction (1,2). The intestinal microbiota, particularly abundant in the colon, has long been known to represent a major source of H_2S in the human gut, where particularly high sulfide levels are reached. Since it is well known that sulfide is a potent inhibitor of respiratory oxidases, such as mitochondrial cytochrome c oxidase (3), we raised the hypothesis that in sulfide-rich environments, like our gut, bacteria can accomplish O2-dependent respiration due to sulfide-insensitive oxidases (4). The hypothesis was tested on Escherichia coli, that has three respiratory oxidases, the heme-copper bo3 enzyme and two bd oxidases (5). The bd-type oxidases are prokaryotic enzymes that are unrelated to heme-copper oxidases and were reported to both promote bacterial virulence and confer resistance to oxidative/nitrosative stress (6,7). Working on the isolated E. coli oxidases, we found that, whereas sulfide is a potent inhibitor of the bo3 enzyme, both bd oxidases are insensitive to sulfide. Moreover, in E. coli respiratory mutants, both O2-consumption and aerobic growth proved to be potently inhibited by sulfide when respiration was sustained by the bo3 oxidase alone, but unaffected even at high sulfide levels when either bd enzyme acted as the only terminal oxidase. Accordingly, wild-type E. coli exhibited sulfide-insensitive respiration and growth under O2-limiting conditions favoring the expression of bd oxidases. Altogether these unprecedented results show that bd oxidases enable sulfide-resistant O2-consumption and growth in E. coli and possibly other bacteria. The physiological significance and potential impact of this discovery are discussed.

References:

1. Shatalin K., Shatalina E., Mironov A., Nudler E. H_2S: a universal defense against antibiotics in bacteria. Science. 2011 Nov 18;334(6058):986-90.

2. Carbonero F., Benefiel A.C., Alizadeh-Ghamsari A.H. Microbial pathways in colonic sulfur metabolism and links with health and disease. Front Physiol. 2012 Nov 28;3:448.

3. Petersen L.C. et al. The effect of inhibitors on the oxygen kinetics of cytochrome c oxidase. Biochim Biophys Acta. 1977 May 11;460(2):299-307.

4. Forte E., Borisov B. Falabella M., Colaço H.G., Tinajero-Trejo M., Poole R.K., Vicente J.B., Sarti P., Giuffre A. Fresh air in the gut: cytochrome bd oxidase promotes sulfide-resistant bacterial respiration and growth. 2016. Submitted.

5. Poole R.K. and Cook G.M. et al. Roles of respiratory oxidases in protecting Escherichia coli K12 from oxidative stress. Antonie Van Leeuwenhoek. 2000 Jul;78(1):23-31.

6. Giuffrè A., Borisov V.B., Arese M., Sarti P., Forte E. Cytochrome bd oxidase and bacterial tolerance to oxidative and nitrosative stress. Biochim Biophys Acta. 2014 Jul;1837(7):1178-87.

7. Borisov V.B., Forte E., Siletsky S.A., Arese M., Davletshin A.I., Sarti P., Giuffrè A. Cytochrome bd protects bacteria against oxidative and nitrosative stress: a potential target for next-generation antimicrobial agents. Biochemistry (Mosc). 2015 May;80(5):565-75.

Mitochondrial-targeted Hydrogen sulfide release by AP39 improves Renal Graft Function and Survival following prolonged cold storage and transplantation and protects renal epithelial cells from *in vitro* Cold Hypoxia/Hypercapnia and Re-Oxygenation Injury

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Organ procurement is inherently associated with ischemia-reperfusion injury (IRI), resulting from loss and subsequent restoration of blood flow, which is detrimental to short- and long-term graft function and survival (1-3). AP39 is a novel mitochondrial-targeted H_2S donor that has shown protective effects during renal epithelial cell oxidative stress (4,5). We postulated that targeting of H_2S release to mitochondria with AP39 would improve the potency of H_2S-mediated protection during in vitro cold hypoxia/hypercapnia and reoxygenation (H/R) injury compared to non-specific H_2S release by GYY4137. We also hypothesized that treatment of donor kidneys with AP39 during prolonged cold storage could mitigate IRI-induced graft injury and improve graft function and survival following syngeneic renal transplantation (RTx).

Rat kidney epithelial cells (NRK-52E; ATCC) were exposed to an in vitro model of cold H/R injury that mimics cellular conditions during in vivo cold IRI. Control cells were cultured in DMEM + 5% fetal bovine serum (FBS) at 37 ºC in room O2 and 5% CO2. Experimental cells were treated with either phosphate-buffered saline (PBS) alone or PBS plus varying concentrations of GYY4137 or AP39 and exposed to cold (10 ºC) hypoxia/hypercapnia (0.1% O2/15% CO2) for 24 hours. Cells were then re-oxygenated for 24 hours in conditions identical to control cells and viability was assessed via flow cytometry using Annexin-V/7-AAD staining, indicating apoptosis and necrosis, respectively. To assess protective effects of AP39 against renal graft IRI, Lewis rats underwent bilateral native nephrectomy and

subsequent RTx with syngeneic donor kidneys flushed with either University of Wisconsin preservation solution (UW group; n=5) or UW + 200 nM AP39 (AP39 group; n=3) and stored for 24 hours at 4°C in the same solution. Sham surgeries (midline incision only; n=5) were also performed and animals were monitored for 14 days to assess graft function and survival.

Cells treated with PBS during in vitro cold H/R injury exhibited significantly decreased ($p<0.001$) viability compared to control (normoxic) cells. Treatment of cells with 200 nM and 400 nM AP39 and 400 µM GYY4137 significantly improved ($p<0.05$) viability compared to PBS, while cells treated with 100 nM, 200 nM, 400 nM and 100 µM GYY4137 exhibited significantly decreased ($p<0.05$) viability compared to 400 nM AP39. Treatment of renal grafts with AP39 significantly improved ($p<0.05$) survival, markedly decreased serum creatinine and significantly decreased ($p<0.05$) tubular necrosis scores compared to UW.

Targeting of H_2S release to mitochondria during in vitro cold H/R injury treatment substantially improved the potency of its protective effects and can also mitigate in vivo IRI associated with prolonged cold storage, improving subsequent graft function and survival. Mitochondria-specific H_2S treatment could represent a novel and cost-effective method of protecting organs during transplantation and improving clinical transplant outcomes.

References:

1. Wilhelm M.J., Pratschke J., Laskowski I. et al. Ischemia and reperfusion injury. Transplant Rev. 2003;17:140.

2. Salahudeen A.K., Haider N., May W. Cold ischemia and the reduced long-term survival of cadaveric renal allografts. Kidney Int. 2004;65:713.

3. Quiroga I., McShane P., Koo D.D. et al. Major effects of delayed graft function and cold ischaemia time on renal allograft survival. Nephrol Dial Transplant. 2006;21:1689.

4. Le Trionnaire S., Perry A., Szczesny B., et al. The synthesis and functional evaluation of a mitochondriatargeted hydrogen sulfide donor, (10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5-yl)phenoxy)decyl) triphenylphosphonium bromide (AP39). Med. Chem. Commun. 2014;(5):728.

5. Ahmad A., Olah G., Szczesny B. et al. AP39, A Mitochondrially Targeted Hydrogen Sulfide Donor, Exerts Protective Effects in Renal Epithelial Cells Subjected to Oxidative Stress in Vitro and in Acute Renal Injury in Vivo. Shock. 2016;45(1):88.

Design and Synthesis of Non Covalent CSE Inhibitors evaluated by Hybrid Methods

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Hydrogen sulfide is an essential catabolite that intervenes in the pathophysiology of several diseases from hypertension to stroke, diabetes and pancreatitis (1,2). It is endogenously synthesized mainly by two pyridoxal-5′-phosphate-dependent enzymes involved in L-cysteine metabolism: cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) (3). A third pathway that catalyses the production of H_2S from L-Cys via the combined action of 3-mercaptopyruvate sulfurtransferase and cysteine aminotransferase has also been described (4). This pathway is less well characterized and its role in determining the H_2S levels in tissues still poorly understood.
Research in this field is currently impaired by the lack of pharmacological tools such as selective enzymatic inhibitors that could target specifically only one of these pathways.

We used a novel approach based on a hybrid method that includes drug design, synthetic biology, metabolomics and pharmacological assays to rationally design a new inhibitor selective for the CSE enzyme. The identification of this compound, embodying the structural features of both cysteine and DL-propargylglycine, and inhibiting CSE by a non covalent mechanism, opens new frontiers towards a better understanding of the role of CSE over CBS in the pathophysiology of diseases where a role for the H_2S pathway has been proposed. On these basis, the development of a novel series of compounds, derived from the here described lead, has been undertaken in order to get a complete SAR study.

References:

1. Wallace J.L., Wang R. Hydrogen sulfide-based therapeutics: exploiting a unique but ubiquitous gasotransmitter Nature Reviews 2015;14,329-345.

2. Caliendo G., Cirino G., Santagada V. Wallace J. L. Synthesis and biological effects of hydrogen sulfide (H_2S): development of H_2S-releasing drugs as pharmaceuticals. J. Med. Chem. 2010;53,6275-6286.

3. Kamoun P. Endogenous production of hydrogen sulfide in mammals. Amino Acids. 2004;26,243-254.

4. Mikami Y., Shibuya N., Kimura Y. Nagahara N., Ogasawara Y., Kimura H. Thioredoxin and dihydrolipoic acid are required for 3-mercaptopyruvate sulfurtransferase to produce hydrogen sulfide. Biochem. J. 2011;439,479-485.

A Proteomic Approach for the Identification of Persulfides in Mammalian Cells

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Cysteine residues of proteins are preferred targets for modifications mediated by reactive oxygen species (ROS) and reactive nitrogen species (RNS) which can lead to dramatic changes in the stability, function, activity and localization of proteins (1). Recently, H_2S was discovered as an important signaling molecule with high therapeutic potential in inflammation, in the cardiovascular system and in cell growth (2). However, its exact mode of action is poorly understood. It is believed that not the gaseous form of H_2S but rather polysulfides can interact with thiols forming persulfides (R-S-SH) and thereby changing the properties of a protein (3). However, due to its ambivalent chemical features it is hard to investigate such a modification on a protein level. On the one hand, persulfides show a similar reactivity towards electrophiles like thiols. On the other hand, they behave similar to other cysteine oxidations in a biotin switch assay. We therefore developed a mass spectrometric based method we named (q)perS-SID (quantitative persulfide site identification) which allows the specific enrichment, isolation and quantification of persulfides on a peptide level. We could show that the diverse H_2S donors Na_2S_4, Na_2S, NaSH and GYY4137 show different potencies to induce persulfides. Bioinformatical assessment of the data revealed that H_2S affects all subcellular compartments and various cellular processes. Furthermore, it seems that negatively charged amino acids appear more frequently in proximity to cysteines forming persulfides. Using PKM2 as a model protein we could confirm our proteomic data showing that persulfide formation at the cysteine residues C49, C152, C358 and C474 leads to its inhibition. Taken together, the identification of persulfides on a proteome scale may help to better understand the biology of H_2S and explain the protective effects of H_2S in several diseases.

References:

1. Paulsen C.E., Carroll K.S. Cysteine-mediated redox signaling: chemistry, biology, and tools for discovery. Chem Rev. 2013;113(7):4633-79. doi: 10.1021/cr300163e.

2. Kimura H. Hydrogen Sulfide and Polysulfides as Biological Mediators. Molecules. 2014;19:16146-16157. doi: 10.3390/molecules191016146.

3. Greiner R., Pàlinkàs Z., Basell K., Bacher D., Antelmann H., Nagy P., Dick T.P. Polysulfides link H_2S to protein thiol oxidation. Antioxid Redox Signal. 2013;19(15):1749-65. doi: 10.1089/ars.2012.5041.

POSTERS

Development of a novel chemiluminescent assay for hydrogen sulfide and its application

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Summary: Hydrogen sulfide (H2S) is attracting attention as one of three endogenously generated gaseous signaling compounds, the others being carbon monoxide and nitric oxide. The hydrogen sulfide in live cells is generated by the following three enzymes: cystathionine β-synthase; cystathionine γ-lyase; and 3-mercaptopyruvate sulfurtransferase. These enzymes are involved in neurotransmitter regulation and vasodilation. However, hydrogen sulfide, the odorous component of waste and sewage, is a toxic gas; therefore, a highly sensitive and specific method for monitoring H2S is desired in order to protect human health and the environment. Hydrogen sulfide is generally measured by gas chromatography, but this method requires special equipment. Fluorescent probes for hydrogen sulfide have also been recently developed as a simpler method. In order to analyze hydrogen sulfide rapidly and sensitively, we have developed a novel method using lucigenin chemiluminescence in the presence of copper ion (II).

Method: Assay method: Lucigenin chemiluminescent solution (0.2 mL: 5 μmol/l copper chloride (II), 0.04 mg/mL lucigenin, 0.1 mg/mL TritonX-100) was added to Na2S solution (20 μl) diluted with phosphate buffer (pH 11.7). Chemiluminecence intensity was measured using an Aloka luminescence reader (Aloka Co. Japan) (waiting time, 10 s; integration time, 10 s).

Results and Discussion: This is a novel chemiluminescence method based on the principle that light is emitted by metal ions and hydrogen sulfide in the presence of lucigenin. The effects of several metal ions (copper (II), copper (I), zinc, magnesium and aliminium) were studied. Intense luminescence was generated with copper (II). Reactive oxygen involved in this chemiluminescent reaction was analyzed using ESR by the addition of a scavenging enzyme, SOD. An ESR signal was observed in the presence of lucigenin. Lucigenin was essential for the generation of active oxygen. With the addition of

both catalase and SOD, this signal essentially disappeared. This result indicates that the radical species is a superoxide anion. Next the effects of pH, lucigenin, copper (II) and phosphate salt concentration were examined to determine the optimal conditions. The results are shown in the Method. Under the above conditions, a standard curve of Na2S shows 1 μmol / 1 (20 pmol / assay) ~ 10 mmol / 1 (20 nmol /assay), and reproducibility was from 1.5 to 11.7% (n = 7), with 6.0& as the mean. The specificity of the methods was examined using cysteine and glutathione as SH compounds. When compared to sodium sulfide standards at the same concentration, the emission intensity was 2.9% and 4.1%, respectively, for cysteine and glutathione. Further, by adding maleic imide to the luminescent reagent, the specificity was able to be improved. Thus, this method was found to show high specificity for Na2S. Currently, we are investigating the application of this methodology to biological samples and environmental studies.

Pro-apoptotic and anti-tumoral activities of natural H_2S donors in melanoma.

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Melanoma is the most common form of skin cancer and it is responsible for the majority of skin cancer deaths. While early-stages melanoma can usually be effectively treated with surgery, more advanced tumors have a high metastatic potential and are notoriously resistant to conventional cancer therapies such as radiation and chemotherapy. Despite significant advances in understanding of melanoma biology and pathogenesis, and the recent success in developing targeted therapies for melanoma (Shtivelman E. et al. 2014, Tsao H. et al. 2012), the prognosis of the disease remains poor, therefore the search for new agents for its treatment is of great importance. We have recently demonstrated that the metabolic pathway l-cysteine/CSE/H_2S is involved in human melanoma progression (Panza et al., 2015). Therefore, the aim of our study was to evaluate the possible anti-tumoral effect of natural H_2S donors. *Ferula assa-foetida L.* is a major source of asafoetida, a foul-smelling gum-resin of dietary and medicinal relevance. Asafoetida is characterized by an unpleasant sulfurous odor, reminiscent of garlic, rotten meat and sweat (Mahendra P. and Bisht S. 2012). In this study, we tested the potential effect of several vinyl disulfide compounds, isolated and purified from asafoetida, on the proliferation of human melanoma cell lines. Toward this goal, the effects of the vinyl disulfides were evaluated in vitro on four different human melanoma cell lines: A375, SK-Mel-5, SK-Mel-28 and PES43. Among all the compounds tested the most effective in suppressing proliferation of melanoma cells resulted the RTFA16C compound (IC50 51,4 µM). Interestingly, the largest degree of cell proliferation inhibition was

observed in melanoma metastatic cells PES43 (38%, 61% and 71%, respectively; p<0,001, n=3) exposed to increasing doses of RTFA 16C (10-30-100µM) for 72h. Thus, this cell line was selected for the subsequent molecular studies. Cytofluorimetric analysis with annexin V/PI staining demonstrated that the anti-proliferative effect of RTFA 16C was due to its ability to induce apoptosis in PES43 cells. Western blot analysis further proved that this compound caused a time-dependent activation of Caspase 3 and the cleavage of its substrate poly (adenosine diphosphate-ribose) polymerase (PARP). The apoptotic machinery can be controlled, at least in part, by NF-κB, which regulates transcription of the Bcl-2 family members (Ben-Neriah and Karin, 2011). Several reports have shown that in melanoma the constitutive activation of NF-κB confers tumor survival capacity and avoidance of apoptosis (Ueda and Richmond, 2006). Thus, we have hypothesized that the RTFA 16C induction of apoptosis was associated with suppression of NF-κB activation. Western blot analysis carried out on the nuclear extracts of PES43 cells incubated with RTFA 16C (30µM) for 3-6-24h revealed a time-dependent reduction of nuclear translocation and activation of p65. Moreover, this effect was also supported by the finding that the treatment with RTFA 16C decreased the expression of the anti-apoptotic proteins c-FLIP, XIAP and Bcl-2, that are transcriptionally regulated by NF-κB. In order to better define the mechanism through which this latter effect is achieved, we investigated the possible involvement of the MAPK/ERK and PI3K/AKT pathways, two of the most frequently deregulated pathways in melanoma (Hodis et al., 2012). Western blot analysis revealed that the treatment of PES43 cells with RTFA 16C (30µM) inhibited the phosphorylation and activation of both AKT and ERK proteins at the time points considered (3-6-24h). Invasivity assay carried out on PES43 cells incubated with RTFA 16C 10 and 30 µM for 16h resulted in a significant inhibition of cell invasion. Finally, to corroborate these results obtained in vitro, we injected B16/F10 mouse melanoma cells into tail veins of C57BL/6 mice to induce lung metastasis. In these mice, the RTFA 16C (50mg/kg, orally administrated) significantly reduced metastatic foci of lung surface when compared to control group. In conclusion, all these findings suggest natural H_2S-donors as new potential agents in the treatment of

human metastatic melanoma and represent a very promising strategy to improve the fight against cancer.

References:

1. Ueda Y, Richmond A. NF-kappaB activation in melanoma. Pigment Cell Res. 2006 Apr;19(2):112-24.

2. Ben-Neriah Y, Karin M. Inflammation meets cancer, with NF-κB as the matchmaker. Nat Immunol. 2011 Jul 19;12(8):715-23.

3. Tsao H, Chin L, Garraway LA, Fisher DE. Melanoma: from mutations to medicine. Genes Dev. 2012 Jun 1;26(11):1131-55. doi: 10.1101/gad.191999.112

4. Hodis E., Watson I.R., Kryukov G.V., Arold S.T., Imielinski M., Theurillat J.P., Nickerson E., Auclair D., Li L., Place C, Dicara D., Ramos A.H. et all. A landscape of driver mutations in melanoma. Cell. 2012 Jul 20;150(2):251-63.

5. Mahendra P., Bisht S. Ferula asafoetida: Traditional uses and pharmacological activity. Pharmacogn Rev. 2012 Jul;6(12):141-6.

6. Shtivelman E., Davies M.Q., Hwu P., Yang J., Lotem M., Oren M, Flaherty KT, Fisher DE. Pathways and therapeutic targets in melanoma. Oncotarget. 2014 Apr 15;5(7):1701-52.

7. Panza E., De Cicco P., Armogida C., Scognamiglio G., Gigantino V., Botti G., Germano D., Napolitano M., Papapetropoulos A., Bucci M., Cirino G., Ianaro A. Role of the cystathionine γ lyase/hydrogen sulfide pathway in human melanoma progression. Pigment Cell Melanoma Res. 2015 Jan;28(1):61-72.

Broad Range Antiviral Activity Of Hydrogen Sulfide

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Background: Hydrogen sulfide (H_2S) is an important endogenous gaseous mediator that has been recently the focus of intense investigation, leading to supportive evidence that it plays an important role in vasoactive, cytoprotective and anti-inflammatory cellular responses. Recently, we have made the critical observation that H_2S has a protective role in paramyxovirus infection by modulating innate inflammatory responses and viral replication [1]. It is not known whether H_2S treatment affects replication of other viruses. In this study we tested the antiviral and anti-inflammatory activity effect of the slow-releasing H_2S donor GYY4137 on four different families of enveloped viruses.

Methods: The following in vitro models of infection were used: family *orthomyxoviridae,* genera *influenza virus A* (H1N1 and H3N2) and *influenza virus B*; family *filoviridae,* genus *ebolavirus* (EBOV-eGFP), family *flaviviridae,* genus *Flavivirus* (RSSEV); family *bunyaviridae,* genus *nairovirus* (CCHFV) and genus *phlebovirus* (RVFV). Infected cells were treated with GYY4137 (Sigma) at 5-10 mM one hour post-infection. Viral loads were assessed in the supernatants and cells at various times post-infection using virus-specific assays. Viral proteins

and RNA were studied by western blotting and real-time PCR. Cytokine and chemokine production was investigated using commercially available ELISA kits and Bioplex assay (Biorad). The effect of GYY4137 on signaling pathways was studied by western blotting of fractionated cellular lysates.

Results: GYY4137 significantly reduced viral replication of all tested viral families. In the case of influenza infection, GYY treatment was associated with decreased expression of several viral proteins, suggesting an inhibition of an early step of replication. The anti-viral activity coincided with the broad decrease in the production of cytokines and chemokines including RANTES, IL-8 and IP10, which was associated with reduced nuclear levels of transcription factors belonging to the NF- κB and IRF families in response to influenza infection.

Conclusions: Treatment with GYY4137 showed significant antiviral activity against a broad range of enveloped viruses, resulting in inhibition of pro-inflammatory cellular signaling and mediators production.

References:

1. Li H., Ma Y., Escaffre O., Ivanciuc T., Komaravelli N., Kelley J.P., Coletta C., Szabo C., Rockx B., Garofalo R.P., Casola A. J Virol. 2015;10:5557-68.

Detrimental effects of gut microbiota-derived hydrogen sulfide on colonic epithelial cells and adaptive responses to a high-protein diet in rats

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Gut microbiota impacts human health and disease through metabolite production. Among them, hydrogen sulfide (H_2S) is produced by intestinal bacteria from sulfur-containing luminal compounds (dietary or host-derived). H_2S concentration in the large intestine content is in the millimolar range (Macfarlane et al., 1992) and several studies reported high fecal concentration of H_2S in inflammatory bowel diseases and colorectal cancer patients (Carbonero et al., 2012). The first aim of the present study was to determine *in vivo* the effects of an increased exposure to luminal H_2S on colonic epithelial cells. We used a model of intra-colonic instillation of NaHS (H_2S donor) or PBS in anesthetized rats. We found that 1-hour instillation with NaHS 1.5 mM induced the expression of pro-inflammatory genes (inducible nitric oxide synthase and interleukin-6) while genes implicated in intestinal inflammation resolution (interleukin-10 and transforming-growth factor β) were not modified by the treatment. Although low millimolar concentration of NaHS inhibited the oxygen consumption of isolated rat colonocytes, NaHS intra-colonic instillation did not modify the respiration of colonocytes but induced the expression of hypoxia-inducible factor 1-α gene. These results suggest that NaHS reversibly disrupted mitochondrial respiration in rat colonocytes. Moreover, we observed that nitric oxide jeopardized the mitochondrial detoxification of H_2S. In contrast with previous reports (Attene-Ramos et al., 2010), we found that H_2S was not genotoxic in colonocytes. Then, we

hypothesized that a high-protein diet (HPD) would modify H_2S metabolism in rat colon since this dietary intervention has been shown to increase amino-acid supply to gut bacteria (Magee et al., 2001). Sulfidogenic microbial pathways were not modified by the HPD in rats but total sulfide in large intestine contents was more than doubled (measured by GS-MS). However, sulfide concentration was not changed (around 0.4 µmol/g) due to increased colon luminal bulk in HPD animals. In addition, the HPD induced an increase in colonocyte gene expression of sulfide quinone reductase, an enzyme involved in H_2S detoxification. In conclusion, excessive exposure of luminal H_2S triggers pro-inflammatory response together with an hypoxia-like state in rat colon epithelium. We also found that the presence of nitric oxide (a hallmark of intestinal inflammation) hampers H_2S detoxification, possibly potentiating the deleterious effects of this bacterial metabolite. Despite HPD increases sulfide production by the microbiota, colonic adaptive responses prevent an increase in sulfide concentration that would be deleterious for colonocytes.

References:

1. Macfarlane G.T., Gibson G.R., Cummings J.H. Comparison of fermentation reactions in different regions of the human colon. J. Appl. Bacteriol. 1992;72, 57–64.

2. Magee E.A., Richardson, C.J., Hughes R., Cummings J.H., 2000. Contribution of dietary protein to sulfide production in the large intestine: an in vitro and a controlled feeding study in humans. Am. J. Clin. Nutr. 72, 1488–1494.

3. Attene-Ramos M.S., Nava G.M., Muellner M.G., Wagner E.D., Plewa M.J., Gaskins H.R. DNA damage and toxicogenomic analyses of hydrogen sulfide in human intestinal epithelial FHs 74 Int cells. Environ. Mol. Mutagen. 5. 20101;304–314. doi:10.1002/em.20546

4. Carbonero F., Benefiel A.C., Gaskins H.R. Contributions of the microbial hydrogen economy to colonic homeostasis. Nat Rev Gastroenterol Hepatol. 2012;9:504–518. doi:10.1038/nrgastro.2012.85.

Role of H_2S in renal ischemia-induced diuresis: H_2S as the oxygen sensor in the kidney.

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Hydrogen sulfide is synthesized in significant amounts in the kidney and has been demonstrated to increase sodium and water excretion [1, 2]. In addition, H_2S is protective in experimental models of various kidney diseases including ischemia-reperfusion injury [3]. The inverse correlation between H_2S concentration and tissue oxygenation was observed in different systems [4]. It is well-known that renal medulla is characterized by very low oxygen pressure [5]. We examined the relationship between H_2S, tissue oxygenation and sodium transport in moderate transient kidney ischemia not associated with structural injury. The study was performed in anesthetized rats. H_2S concentration in renal cortical and medullary compartments was measured by microdialysis method using specific polarographic sensor. The expression and activities of H_2S-synthetizing enzymes cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST) were markedly higher in the renal cortex than in the renal medulla, however, H_2S concentration was about three-fold higher in the interstitial fluid collected from the renal medulla most likely due to much lower oxygen level (pO_2 in the renal cortex and medulla being 76 ± 6 and 16 ± 5 mmHg, respectively). In addition, stigmatellin – the inhibitor of mitochondrial H_2S oxidation – infused into the renal artery markedly increased H_2S concentration in the renal cortex but has no significant effect in the renal medulla. Transient renal ischemia induced by clamping the renal artery for 30 min reduced pO_2 in the cortex and medulla from 76 ± 6 to 27 ± 4 mmHg and from 16 ± 5 to 7 ± 3 mmHg, respectively. In addition, renal ischemia increased H_2S concentration in the renal medulla but not in the cortex. Urinary Na^+ excretion increased after restoration of renal blood flow and this effect was attenuated by CSE inhibitor, propargylglycine. In conclusion: (1) H_2S concentration in the renal medulla is higher than in the cortex due to low oxygen

concentration. (2) H_2S concentration in the renal medulla is sensitive to changes in oxygenation and increases during renal ischemia, (3) H_2S mediates, at least in part, post-ischemic increase in natriuresis.

References:

1. Xia M., Chen L., Muh R.W., Li P.L., Li N. Production and actions of hydrogen sulfide, a novel gaseous bioactive substance, in the kidneys. J Pharmacol Exp Ther. 2009;329(3):1056-62. doi: 10.1124/jpet.108.149963.

2. Ge S.N., Zhao M.M., Wu D.D., Chen Y., Wang Y., Zhu J.H., Cai W.J., Zhu Y.Z., Zhu Y.C. Hydrogen sulfide targets EGFR Cys797/Cys798 residues to induce Na^+/K^+-ATPase endocytosis and inhibition in renal tubular epithelial cells and increase sodium excretion in chronic salt-loaded rats. Antioxid Redox Signal. 2014;21(15):2061-82. doi: 10.1089/ars.2013.5304.

3. Koning A.M., Frenay A.R., Leuvenink H.G., van Goor H. Hydrogen sulfide in renal physiology, disease and transplantation--the smell of renal protection. Nitric Oxide. 2015;46:37-49. doi: 10.1016/j.niox.2015.01.005.

4. Olson K.R. Hydrogen sulfide as an oxygen sensor. Antioxid Redox Signal. 2015;22(5):377-97. doi: 10.1089/ars.2014.5930.

5. Evans R.G., Ince C., Joles J.A., Smith DW, May CN, O'Connor PM, Gardiner BS. Haemodynamic influences on kidney oxygenation: clinical implications of integrative physiology. Clin Exp Pharmacol Physiol. 2013;40(2):106-22. doi: 10.1111/1440-1681.12031.

Investigating the Redox Chemistry of Perthiyl Radicals: An Implication of their Biological Funcitions

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Due to its discovery as a signaling agent in mammalian systems, hydrogen sulfide (H2S) has recently gained much attention among the research community (1). Although H2S has been reported to be involved in many cellular signaling pathways and numerous physiological roles for H2S have been proposed, the exact mechanisms of its biological actions are not yet well defined. Recent work suggests that in fact hydropersulfides (RSSH/RSS-) are responsible for some, if not most, of the biological effects attributed to H2S and rather, H2S production serves as more of a marker or indicator for the presence of hydropersulfide reactivity (2). However, it should be noted that until now, the study of hydropersulfides alone has been difficult for often times their generation requires the presence/use of H2S and oxidized thiol species (4). Therefore, the development of H2S-independent hydropersulfide donors is of great importance. For this reason we, and others have employed the use of protected hydropersulfides (RSSPG), which undergo spontaneous rearrangement for in situ generation of hydropersulfide species (3). Use of such donors has allowed for a more definitive study of the chemistry associated with hydropersulfides and their related redox species. Specifically, focus has been placed on studying the ability of hydropersulfides to serve as one- and two-electron reductants. One-electron oxidation of a hydropersulfide leads to the generation of perthiyl radicals (RSS•), which in comparison to thiyl radicals (RS•), have an enhanced stability through resonance of the unpaired electron with the orbitals of the neighboring sulfur atom. For this reason, examination of the fundamental redox chemistry of hydropersulfides and perthiyl radicals with biologically

relevant species (e.g.O2 and nitrogen oxides) has been undertaken and the results are discussed here.

References:

1. Wang R. Physiological implications of hydrogen sulfide: a whiff exploration that blossomed. Physiol. Rev. 2012;348:791–896.

2. Ida T., Sawa T., Ihara H., Tsuchiya Y., Watanabe Y., Kumagai Y. et al. Reactive cysteine persulfides and S-polythiolation regulate oxidative stress and redox signaling. Proc Natl Acad Sci U S A. 2014 May;111(21):7606–11.

3. Artaud I., Galardon E. A Persulfide Analogue of the Nitrosothiol SNAP: Formation, Characterization and Reactivity. ChemBioChem. 2014 Nov 3;15:2361–4.

4. Vasas A., Dóka É., Fábián I., Nagy P. Nitric Oxide Kinetic and thermodynamic studies on the disulfidebond reducing potential of hydrogen sulfide. Nitric Oxide. Elsevier Inc. 2015;46:93–101.

H_2S Donors selectively up regulate cystathionine-γ Lyase

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Exposure of cells to receptor agonists typically leads to down regulation of their expression, while treatment with antagonists usually results in up regulation of the targeted receptor. Sporadic evidence in the literature has indicated that treatment with H_2S releasing agents leads to an increase in CSE expression. In addition, it has been observed that the effects of H_2S donors are in some instances blocked by CSE inhibitors. The aim of the present study was to systematically investigate whether administration of H_2S donors alters the levels of H_2S producing enzymes. H9c2 differentiated cardiomyocytes and rat heart endocardial cells were exposed to vehicle, Na_2S (100μM), GYY (100μM), thiovaline (1μM), AP-39 (1nM) for 24hr. Such treatment resulted in elevation of mRNA and protein CSE levels. In contrast, no changes in the expression of CBS or 3MST were noted. In line with the notion that Na_2S stimulates cGMP accumulation, we found that up regulation of CSE expression in cardiomyocytes can be blocked by DT-2, a cGMP-dependent protein kinase (PKG) inhibitor. To test whether H_2S also regulates CSE levels in vivo, C57BL/6 male mice were treated with H_2S donors and levels of CSE were determined. We observed that following H_2S donor treatment CSE expression in the aorta, carotid and the heart was increased, confirming our in vitro observations. Our findings raise the possibility that a feed-forward mechanism exists during which exposure to H_2S donors drives CSE expression leading to generation of uncontrolled H_2S amounts that could potentially reach toxic levels. PKG was recently demonstrated to phosphorylate CSE on Ser377 restricting its activity. H9c2 cardiomyocytes were transfected with human CSE and exposed to Na_2S. After such treatment, immunoprecipitated CSE was found to interact with PKG. Using a

phospho-Ser Ab we found an increase in CSE serine phosphorylation following treatment of cells with Na_2S. We next raised a phospho-specific Ab against Ser377 and tested for CSE phosphorylation on this residue in animals treated with Na_2S for 24h. Indeed, H_2S donor-treated mice exhibited an increase of CSE phosphorylation on Ser377 compared to vehicle-treated mice. We conclude that H_2S donors up regulate CSE expression in a PKG-dependent manner and that uncontrolled production of H_2S is prevented by phosphorylation of CSE on Ser377.

Alterations in the cardiovascular system of 3 MST knockout mice

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Most of the endogenously produced H_2S is enzymatically generated by cystathionine beta synthase (CBS), cystathionine gamma lyase (CSE) and 3-mercaptopyruvate sulfur transferase (3-MST). The three H_2S-producing enzymes differ in their sub-cellular localization pattern, regulation, substrate utilization and co-factor requirement. Research on H_2S in the cardiovascular system has focused on CSE, since this enzyme was believed to be the prevalent source for H_2S in the heart and blood vessels. The generation of a CSE KO mouse line and the existence of reasonably selective pharmacological inhibitors for CSE have greatly facilitated preclinical experimentation.

More recently, 3MST has attracted significant attention as a source of H_2S. 3MST is abundantly expressed in blood vessels and the myocardium and can be found both in mitochondria and in the cytosol. However, its contribution to cardiovascular homeostasis has not been addressed, as no useful pharmacological inhibitors exist. Although a global 3MST KO has been created, its cardiovascular phenotype has not been characterized. The aim of the current study was to unravel the role of 3MST in cardiovascular homeostasis. 3MST KO animals exhibited normal body weight, but slightly elevated heart to body weight ratios. Although, heart rate was similar between 3MST and wild-type animals, changes in the ECG were observed. Expression analysis for H_2S-generating enzymes in the myocardium, aorta and carotid artery revealed differential expression of the H_2S-producing enzymes. CBS and CSE remained unaltered in 3MST KO

hearts; in the carotid arteries of 3MST KO CBS levels were reduced. In the aorta, CBS expression was increased, while CSE levels were reduced. We also determined the levels of cGMP-dependent protein kinase (PKG), endothelial nitric oxide synthase (eNOS) and soluble guanylate cyclase (sGC); PKG expression in the heart was elevated, while no changes in the levels of the 3 enzymes were detected in the blood vessels. Systolic blood pressure in 3MST KO mice was not different from that of wild-type control animals. Phenylephrine-induced contractions, as well as L-cysteine – and acetylcholine-induced relaxations of aortic rings were similar between 3MST KO and wild-type controls. Relaxations to NaHS were slightly potentiated in 3MST KO animals.

To test the contribution of 3MST in ischemia/reperfusion injury, mice were subjected to 30 minutes ischemia (LAD ligation) followed by 2 hours of reperfusion and the infarct size was assessed. Interestingly, 3MST KO animals revealed a statistically significant reduction in myocardial infarct size. A similar effect was observed in vitro in H9c2 differentiated cardiomyocytes; cells infected with 3MST adenovirus showed increased cell death when exposed to H_2O_2 compared to the GFP-infected controls. We conclude that 3MST impacts cardiac structure and function and that, unlike CSE, 3MST does not exert a protective effect in the context of I/R injury in the heart.

The Novel Mitochondria-Targeted Hydrogen Sulfide Donor AP123 restores nitric oxide pathway in endothelial cells grown in high glucose environment

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Diabetes represents a major disease associated to severe vascular complications, such as atherosclerosis and diverse forms of angiopathy, that converge in hypertension [1-4]. The mechanism underlying these events have been widely disclosed and involve, among other factors, endothelial nitric oxide synthase (eNOS). In particular, eNOS activity is suppressed in hyperglycaemic conditions, resulting in a reduced NO bioavailability. This change is coupled with increased levels of caveolin-1 (Cav-1), a protein that negatively regulates eNOS function, and to reduced eNOS phosphorylation (p-eNOS) [5,6]. In addition, diabetic conditions are also linked to reduced circulating hydrogen sulfide (H2S) levels [7], further undermining vascular homeostasis with respect to endogenous vasorelaxant response [8] and suggesting that diabetes is a condition of “H2S deficiency” [9]. Here, we wanted to test whether the mitochondria-targeted H2S donor molecules, AP123 and AP39 [10,11], could affect the changes to NO-signalling observed in diabetic conditions. For this purpose, we used an established model of in vitro hyperglycaemia, where bovine aortic endothelial cells (BAEC) were grown in high glucose (HG, 50mM) environment for 3h (Bucci et al., 2004; Bucci et al., 2008). AP123 and AP39 were added at same time as HG induction or 1h later. After incubation, cells were challenged with calcium ionophore A23187 (1µM, 30min) to

stimulate eNOS activation and then collected to perform western blot analysis. Supernatants were used for fluorometric evaluation of nitrite/nitrate (NOx) levels. Our results show that incubation BAEC with AP123 (3h, 1µM), starting at the same time as HG condition, restored NOx levels and only slightly restored eNOS, p-eNOS and Cav-1 expression. More interestingly, AP123 added 1h after HG trigger similarly restored NOx levels, and, in addition, increased expression of eNOS and p-eNOS while reduced Cav-1. In sharp contrast, incubation of BAEC with AP39 (1µM) under identical conditions did not? show significant changes in either NOx levels nor eNOS and Cav-1 expression. Overall, these data highlight that AP123 is more effective than AP39 in restoring NOx levels and eNOS/Cav-1 balance, presumably since the effects of AP39 are known to be NO-independent. These studies strongly suggest that H2S may be a crucial vasculoprotective mediator in diabetic vasculature and that pharmacological supply of H2S by novel H2S-releasing molecules may be useful in counteracting the detrimental effects to the vasculature of "H2S deficiency" and high glucose environment, typical of diabetic state.

References:

1. Graier W.F., Posch K., Fleischhacker E., Wascher T.C., Kostner G.M. Diabetes Res Clin Pract. 1999 Sep;45(2-3):153-60.

2. Jensen-Urstad K.J., Reichard P.G., Rosfors J.S., Lindblad L.E., Jensen-Urstad M.T. Diabetes. 1996 Sep;45(9):1253-8.

3. Schmidt A.M., Yan S.D., Wautier J.L., Stern D. Circ Res. 1999 Mar 19;84(5):489-97.

4. Kennon B., Petrie J.R., Small M., Connell J.M.. Diabet Med. 1999 Jun;16(6):448-58.

5. Bucci M., Roviezzo F., Brancaleone V., Lin M.I., Di Lorenzo A., Cicala C., Pinto A., Sessa W.C., Farneti S., Fiorucci S., Cirino G. Arterioscler Thromb Vasc Biol. 2004 Apr;24(4):721-6.

6. Bucci M., Roviezzo F., Brancaleone V., Di Lorenzo A, Evangelista S, Gori M, Cirino G. Vascul Pharmacol. 2008 Aug-Sep;49(2-3):84-90

7. Whiteman M., Gooding K.M., Whatmore J.L., Ball C.I., Mawson D., Skinner K., Tooke J.E., Shore A.C. Diabetologia. 2010 Aug;53(8):1722-6.

8. Brancaleone V., Roviezzo F., Vellecco V., De Gruttola L., Bucci M., Cirino G. Br J Pharmacol. 2008 Nov;155(5):673-80.

9. Suzuki K., Olah G., Modis K., Coletta C., Kulp G., Gerö D., Szoleczky P., Chang T., Zhou Z., Wu L., Wang R., Papapetropoulos A., Szabo C. Proc Natl Acad Sci U S A. 2011 Aug 16;108(33):13829-34.

10. Le Trionnaire S., Perry A., Szczesny B., Szabo C., Winyard P.G., Whatmore J.L., Wood M.E., Whiteman M. Med. Chem. Commun. 2014;5:728-36.

11. Szczesny B., Modis K., Yanaqi K., Coletta C., Le Trionnaire S., Perry A., Wood M.E., Whiteman M., & Szabo C. Nitric Oxide. 2014;41:120-30.

Cystathionine-b-synthase inhibition for colon cancer: Enhancement of the therapeutic efficacy of aminooxyacetic acid via the prodrug approach: in vitro studies

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Colon cancer cells contain high levels of cystathionine-b-synthase (CBS). Its product, hydrogen sulfide (H2S), promotes the growth and proliferation of colorectal tumor cells in vitro and in vivo [1]. The CBS inhibitor aminooxyacetic acid (AOAA) suppresses the proliferation of colon cancer cells in vitro and reduces tumor growth in vivo [1]. The potency of AOAA in recombinant CBS (IC50: 10 μM) was markedly lower than the potency of the compound as an antiproliferative agent in the colon cancer cell line HCT116 in vitro (IC50: 600 μM). We hypothesized that the difference between enzyme potency and cell-based efficacy may be related to a limited cellular uptake of AOAA. Therefore, we have designed and synthesized an AOAA prodrug (AOAA methyl ester: YD0171) and tested it in vitro and in vivo in various colon cancer models. YD0171 did not inhibit recombinant CBS, but potently inhibited CBS activity in HCT116 cells, consistent with cellular uptake and intracellular cleavage of the prodrug. The antiproliferative effect of YD0171 exceeded the antiproliferative potency of AOAA in HCT116 cells. The esterase inhibitor paraoxon prevented the inhibition of CBS activity by YD0171 in the HCT116 cells. YD0171 suppressed mitochondrial function and glycolytic activity, but did not induce tumor cell necrosis or apoptosis. The antiproliferative effects of YD0171 or AOAA were not associated with cell cycle block, but both compounds induced a lengthening of the G1 phase, a shortening of the G2/M phase, while the S phase was unaffected. Metabolomic analysis in HCT116 cells showed that YD0171 affects multiple pathways of cell metabolism in a manner that was similar to the effect of AOAA; from 1300

metabolites over 200 are affected, including the expected alterations in transsulfuration pathway intermediers. These in vitro data suggest that the prodrug approach, as exemplified by YD0171, may be a potential strategy to increase the antitumor efficacy of AOAA.

References:

1. Szabo C., Coletta C., Chao C., Módis K., Szczesny B., Papapetropoulos A., Hellmich MR. Tumor-derived hydrogen sulfide, produced by cystathionine-β-synthase, stimulates bioenergetics, cell proliferation, and angiogenesis in colon cancer. Proc Natl Acad Sci U S A. 2013;110:12474-9.

Cardio-Protective Effects Of H2S Through Modulation Of Thioredoxin Reductase Activity And Redox Homeostasis

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H2S is known for its beneficial effects under various cardiovascular conditions, including ischemia/reperfusion (I/R) injury, myocardial infarction, cardiac hypertrophy, fibrosis, heart failure amongst others. The understanding of mechanistic basis of such effects has thus attracted the attention of numerous cardiovascular researchers [1]. We here utilized H9c2 cardiac myoblasts and studied the effects of H2S supplementation (sodium hydrosulfide, NaHS) during pathological adreno-receptor stimulation – involving production of ROS messenger during the signaling process. To test the possibility of direct/indirect effects on redox modulation, we studied effects produced by pre- or parallel-treatment with NaHS during adrenergic stress conditions. Time-course microscopic imaging for ROS (employing DCFH-DA) besides flow cytometry analysis, post adrenergic stimulation, suggested higher efficacy of pre-treatment (over parallel treatment) with NaHS. Interestingly, pre-treatment with NaHS, led to increase in the expression and activity of major anti-oxidant enzymes, including Thioredoxin Reductase (TrxR), Glutathione Reductase (GSR) and Catalase. Inhibition of TrxR activity curtailed the beneficial effects of NaHS in our in vitro model system. We also utilized an established rodent model of cardiac stress and hypertrophy [2]. These experiments also yielded evidence for increase in TrxR expression and activity in heart tissue of NaHS pre-treated animals besides significant reduction in ROS, during adrenergic stimulation. Further, multiple morphometric and histological evidences supported amelioration of agonist induced cardiac remodeling in presence of exogenous H2S donor. Taken together, these results highlighted H2S as a key modulator of redox machinery in cardiac cells besides its ability to regulate pathological outcomes during cardiac stress conditions.

References:

1. Banerjee R. Hydrogen sulfide: redox metabolism and signaling. Antioxidants & redox signaling. 2011;15(2):339-41. doi: 10.1089/ars.2011.3912. PubMed PMID: 21275829; PubMed Central PMCID: PMCPMC3118658.

2. Lopert P, Day BJ, Patel M. Thioredoxin reductase deficiency potentiates oxidative stress, mitochondrial dysfunction and cell death in dopaminergic cells. PloS one. 2012;7(11):e50683. doi: 10.1371/journal.pone.0050683. PubMed PMID: 23226354; PubMed Central PMCID: PMCPMC3511321.

3. Santos CX, Anilkumar N, Zhang M, Brewer AC, Shah AM. Redox signaling in cardiac myocytes. Free radical biology & medicine. 2011;50(7):777-93. doi: 10.1016/j.freeradbiomed.2011.01.003. PubMed PMID: 21236334; PubMed Central PMCID: PMCPMC3049876.

4. Seddon M, Looi YH, Shah AM. Oxidative stress and redox signalling in cardiac hypertrophy and heart failure. Heart. 2007;93(8):903-7. doi: 10.1136/hrt.2005.068270. PubMed PMID: 16670100; PubMed Central PMCID: PMCPMC1994416.

5. Singal PK, Khaper N, Palace V, Kumar D. The role of oxidative stress in the genesis of heart disease. Cardiovasc Res. 1998;40(3):426-32. PubMed PMID: 10070480.

6. Stein A, Bailey SM. Redox Biology of Hydrogen Sulfide: Implications for Physiology, Pathophysiology, and Pharmacology. Redox Biol. 2013;1(1):32-9. doi: 10.1016/j.redox.2012.11.006. PubMed PMID: 23795345; PubMed Central PMCID: PMCPMC3685875.

Iminothioethers as a novel class of H2S-donor: gasotransmitter release and vascular effects.

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Hydrogen sulphide (H2S) is now considered an important gasotransmitter exerting a plethora of effects in particular in controlling the homeostasis of the cardiovascular system. Endogenous H2S is mainly produced in various mammalian tissues by specific enzymes, such as cystathionine beta synthase (CBS) and cystathionine gamma lyase (CSE) responsible for metabolizing L-Cysteine (L-Cys) [1]. Blunted levels of endogenous H2S have been found in animal models of many pathological conditions, such as myocardial ischemia, spontaneous hypertension and hypoxic pulmonary hypertension. Therefore, the administration of exogenous H2S may represent an attractive pharmacological strategy. However, the administration of excessively rapid H2S donors, such as NaHS, is not suitable for clinical purposes. In contrast, organic molecules that are endowed with slow H2S releasing properties may have a relevant clinical usefulness [2]. In this study a small library of iminothioethers was synthesised and their H2S-releasing properties were evaluated in vitro, by amperometric detection, both in the absence and in the presence of organic thiols, such as L-Cys. Furthermore, their vasorelaxing properties were assessed in rat aortic rings. The compounds which exerted the better H2S releasing properties (IS-313 and SM-54), were selected for further pharmacological studies: their effect on membrane potential of cultured human aortic smooth muscle cells (HASMCs) was measured by indirect electrophysiology using bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol (DiBac4(3)); the H2S release of these compounds was evaluated in cultured HASMC with the Washington soluble probe-1(WSP-1) which reacts with H2S, by means of spectrofluorimetric recording and confocal microscopy. In the amperometric assay, carried out in PBS buffer (i.e. in the absence of any biological substrate), all the tested compounds showed negligible H2S release in the absence of L-Cys. In

contrast, in the presence of L-Cys, the iminothioethers exhibited clear H2S releasing profiles, strongly influenced by the chemical structure. Consistently, all the iminothioethers showed endothelium-independent vasorelaxing effects on rat aortic rings. SM-54 and IS-313, which showed a better H2S releasing profile, were selected in order to evaluate their effect on membrane potential in HASMC: both compounds evoked membrane hyperpolarization, with a concentration-dependent trend but with different efficacy: IS-313 was more effective than SM-54. In order to evaluate the actual generation of H2S in the biological environment, HASMCs were incubated with WSP-1 and treated with the H2S-donors; in such an experimental set up, both compounds showed time-dependent increasing concentrations of H2S. Interestingly, the confocal microscopy confirmed the intracellular production of H2S in HASMCs treated with both IS-313 and SM-54, indicating that these compounds can easily enter into cells and then release H2S. The results obtained with tested compounds demonstrate that the iminothioether functional group represents a totally new moiety able to release H2S in a slow and constant manner and to produce H2S-mediated biological effects, thus representing a useful tool for pharmacological approaches for those diseases linked to a deficiency of endogenous H2S.

References:

1. Abe K, Kimura H. “The possible role of hydrogen sulfide as an endogenous neuromodulator.” J Neurosci. 1996 Feb;16(3):1066-71.

2. Szabó C. “Hydrogen sulphide and its therapeutic potential.” Nat Rev Drug Discov. 2007 Nov;6(11):917-35.

Effects of hydrogen sulfide donors on pruritus induced by an agonist of type-2 protease activated receptors (PAR-2) in mice.

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Pruritus is the most common symptom of cutaneous diseases and anti-histamines are the usual treatment; however, anti-histamine-resistant pruritus is very common in some clinical settings, thus reflecting the need to target alternative pathways. Considering that the intradermal (i.d.) injection of the peptide SLIGRL-NH2 (an agonist of the type-2 protease activated receptor - PAR-2) evokes a scratching behavior in mice (Shimada et al., 2006), and that hydrogen sulfide (H2S) donors can inhibit histamine-mediated itching (Rodrigues et al., 2013), in the present study, we investigated the effects of H2S donors on the acute scratching behavior mediated by the activation of PAR-2 in mice, as well as some of the possible pharmacological mechanisms involved. The experimental protocol was approved by the local Ethics Committee for Animal Experimentation (CEUA - ICB/USP; n° 100, fls. 09, livro 03/2013). Male C57BL/6 mice (7-10 wk-old) received an i.d. injection of SLIGRL-NH2 (40 nmol/site) into the dorsal neck region. GYY4137 and NaHS (slow-releasing and spontaneous H2S donors, respectively) were injected either concomitantly with or 30 min before SLIGRL-NH2 into the same site. The itching response was assessed by the number of scratching bouts during the 40 min following SLIGRL-NH2 injection. The i.d. injection of SLIGRL-NH2 (8-80 nmol/site) caused a dose-dependent scratching, with a peak response observed at 10 min and a return to baseline within 30 min; pre-treatment with the histamine H1 receptor antagonist pyrilamine (30 mg/kg, i.p.) did not inhibit this behavior. Co-injection of SLIGRL-NH2 (40 nmol/site) with either the slow-releasing H2S donor GYY4137 (1 and 3 nmol/site) or the spontaneous donor NaHS (0.3 and 1 nmol/site) resulted in significantly reduced responses. Pre-

treatment with NaHS, but not with GYY4137, also resulted in significant reduction of the scratching behavior. Co-treatment with the KATP channel blocker glibenclamide (200 nmol/site) abolished the antipruritic effects of H2S. The simultaneous injection of the non-enzymatic nitric oxide (NO) donor sodium nitroprusside (10 nmol/site) significantly reversed the antipruritic effect of H2S; however, this effect was independent of soluble guanylyl cyclase stimulation, since co-treatment with the specific inhibitor ODQ (3-30 g/site) had no significant effects on the antipruritic actions of H2S. The TRPA1 antagonist HC-030031 (20 □g/site) significantly reduced SLIGRL-NH2-induced scratching behavior; however the scratching behavior induced by the TRPA1 agonist AITC (1000 nmol/site) was not affected by H2S. Our data show that pruritus secondary to PAR-2 activation can be alleviated by H2S, which acts through opening KATP channels and also involves NO in a cGMP-independent manner. Furthermore, TRPA1 receptor may mediate the pruritus induced by activation of PAR-2, but H2S does not interfere with this pathway. In conclusion, these results provide support for the development of new treatments for pruritus, mainly those aiming situations where anti-histamines are devoid of significant efficacy.
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References:

1. Shimada et al. Eur J Pharmacol. 2006 Jan 20;530(3):281-3.

2. Rodrigues et al. Nitric Oxide. 2013;31(Suppl 2),S54–S55.

The Interaction of L-Cysteine/Hydrogen Sulfide Pathway and Muscarinic Acetylcholine Receptors (mAChRs) in Mouse Corpus Cavernosum

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Introduction: The relaxant effect of exogenous hydrogen sulfide (H2S) has been demonstrated in mouse6, rat5, rabbit2, primate1 and human4 corpus cavernosum (CC) tissues. Also, it has been reported that H2S synthesizes endogenously from L-cysteine substrate in cavernosal tissues and suggested that L-cysteine/H2S pathway may regulate penile erection4. The deletion of CSE enzyme markedly diminished endothelium-dependent vasorelaxation induced by cholinergic agonist methacholine, and the augmentation of H2S levels via methacholine treatment of cultured endothelial cells was blocked by cholinergic antagonist in mice3. In addition, we previously shown that atropine reduced exogenous H2S-induced relaxations in mouse CC and we suggested that exogenous H2S may produce its relaxant response through the muscarinic receptors6. However, the mechanism of this relaxant effect is still unclear. The aim of this study was to investigate the possible interaction of L-cysteine/H2S pathway and muscarinic acetylcholine receptors (mAChRs) in the mouse CC.

Method: In the present study, the relaxant responses to L-cysteine (endogenous H2S substrate; 10-3 M), sodium hydrogen sulfide (NaHS; exogenous H2S; 10-6–10-3 M) and acetylcholine (10-6 M) were obtained in isolated mouse CC tissues. Firstly, the effects of propargylglycine (PAG, 10–2 M) or aminooxyacetic acid (AOAA, 10–3 M), inhibitors of H2S synthase enzymes cystathionine-gamma-lyase (CSE) and cystathionine-β-synthase (CBS) respectively, on the relaxant response to L-cysteine and acetylcholine were investigated. The role of non-selective mAChr antagonist atropine (5x10-5 M),

selective M1 receptor antagonist pirenzepine (5x10-6 M), selective M2 receptor antagonist AF-DX (10-6 M) and selective M3 receptor antagonist 4-DAMP (10-6 M) were investigated on the relaxant responses to L-cysteine, exogenous H2S and acetylcholine in isolated mouse CC tissues which were pre-contracted by phenylephrine (5x10-6 M).

Result: The relaxant responses to L-cysteine were significantly reduced by PAG, but not by AOAA. On the other hand, the relaxant responses to acetylcholine were not influenced by PAG, but were significantly increased by AOAA. Atropine, pirenzepin and 4-DAMP significantly reduced the relaxant response to L-cysteine. However, AFDX-116 did not cause a significant inhibition on the relaxant response to L-cysteine. Exogenous H2S-induced relaxant response was significantly reduced by atropine, pirenzepin, 4-DAMP and AFDX-116. Furthermore, atropine, pirenzepin and 4-DAMP significantly inhibited the relaxant response to acetylcholine. Whereas AFDX-116 did not affect these relaxant responses.

Discussion and conclusion: We conclude that L-induced relaxant response is mediated via activation of CSE, and mAChRs M1 and M3 but not M2 receptors contributes to relaxant effect of L- cysteine in mouse CC. Also, mAChRs plays a role in exogenous H2S-induced relaxation in this tissue.

Key words: acetylcholine, corpus cavernosum, hydrogen sulfide, L-cysteine, muscarinic receptors, NaHS

References:

1. Srilatha B., Adaikan P.G., Moore P.K. Possible role for the novel gasotransmitter hydrogen sulphide in erectile dysfunction--a pilot study. *Eur* J Pharmacol. 2006;535(1-3):280-2.

2. Srilatha B., Adaikan P.G., Li L., Moore P.K. Hydrogen sulphide: a novel endogenous gasotransmitter facilitates erectile function. J Sex Med. 2007;4(5):1304-11.

3. Yang G, Wu L., Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, Snyder SH, Wang R. H2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. Science. 2008;322(5901):587-90.

4. D'Emmanuele di Villa Bianca R., Sorrentino R., Maffia P., Mirone V., Imbimbo C., Fusco F., De Palma R., Ignarro L.J., Cirino G. Hydrogen sulfide as a mediator of human corpus cavernosum smooth-muscle relaxation. Proc Natl Acad Sci USA. 2009;106(11):4513-8.

5. Ghasemi M., Dehpour A.R., Moore K.P., Mani A.R. Role of endogenous hydrogen sulfide in neurogenic relaxation of rat corpus cavernosum. Biochem Pharmacol. 2012;83(9):1261-8.

6. Aydinoglu F., Ogulener N. Characterization of relaxant mechanism of H_2S in mouse corpus cavernosum. Clin Exp Pharmacol Physiol. 2016;[Epub ahead of print]. doi: 10.1111/1440-1681.12554.

Modulation of the inflammatory response by hydrogen sulfide in a model of colitis-associated cancer

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Chronic inflammation is now accepted as a key component of tumor progression and development (Hanahan and Weinberg, 2011) and inflammatory bowel diseases (IBDs) are considered major risk factors for colorectal cancer (Eaden et al., 2001). Several evidences link H_2S to colonic nociception, IBDs and colorectal cancer. The exact mechanisms and pathways by which H_2S exerts its multitude of effects are not yet fully understood, but its involvement in physiological and pathophysiological conditions of the colon is becoming evident and several studies support the anti-inflammatory activity of H2S in experimental colitis (Xu et al., 2005; Fiorucci et al., 2007; Wallace et al., 2009). The aim of the present study was to evaluate the role of H2S during the pathogenesis of colitis-associated cancer induced by infection with the intestinal bacteria Helicobacter hepaticus (Hh).

Infection of 129SvEvS6/Rag2-/- mice with Hh for three consecutive days led to the development of severe intestinal inflammation, characterized by marked epithelial cell hyperplasia, extensive inflammatory infiltrates and goblet cell depletion that is entirely dependent on innate immunity. Sustained inflammation in the colon of Hh-infected mice was correlated with a marked reduction (**$P<0.01$) of H2S synthesis at 3 and 6 weeks. Analysis of both protein and mRNA of H2S synthesizing enzymes cystathionine-beta-synthase (CBS) and cystathionine-gamma-lyase (CSE) was carried out on colon samples from healthy and Hh-infected mice at 6 weeks to evaluate the contribution of these enzymes in H2S production during Hh inducing

colitis. The results demonstrated that both enzymes were constitutively expressed in the colon of healthy mice and that CBS, but not CSE, was significantly reduced during colitis development. Finally, we found that enhancement of H2S levels in Hh-infected mice, obtained by administration of L-cysteine (1g/Kg) or diallyl trisulfide (DATS) (50mg/Kg), resulted in a significant reduction of inflammation in the distal part of the colon (*P<0.05). In conclusion, these results demonstrate that H2S has a protective role in a model of colitis driving colorectal cancer.

References:

1. Eaden J.A., Abrams K.R., Mayberry J.F. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut. 2001 Apr;48(4):526-35.

2. Xu X.M., Yu J.P., He X.F., Li J.H., Yu L.L., Yu H.G. Effects of garlicin on apoptosis in rat model of colitis. World J Gastroenterol. 2005 Aug 7;11(29):4579-82.

3. Fiorucci S., Orlandi S., Mencarelli A., Caliendo G., Santagada V., Distrutti E., Santucci L., Cirino G., Wallace J.L. Enhanced activity of a hydrogen sulphide-releasing derivative of mesalamine (ATB-429) in a mouse model of colitis. Br J Pharmacol. 2007 Apr;150(8):996-1002.

4. Wallace J.L., Vong L., McKnight W., Dicay M., Martin G.R. Endogenous and exogenous hydrogen sulfide promotes resolution of colitis in rats. Gastroenterology. 2009 Aug;137(2):569-78.

5. Hanahan D., Weinberg R.A. Hallmarks of cancer: the next generation. Cell. 2011 Mar 4;144(5):646-74.

Beneficial effects of the slow-releasing hydrogen sulfide donor GYY-4137 on the acute carrageenan-induced synovitis of the temporomandibular joint in rats

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Temporomandibular joint (TMJ) disorders are usually associated with inflammation and pain. In previous studies, we demonstrated that hydrogen sulfide (H2S) exerts beneficial effects on nociception and inflammation secondary to knee joint synovitis induced by carrageenan (CGN) in rats (Ekundi-Valentim et al., 2010, 2013). GYY-4137 is a slow H2S-releasing compound that has shown promising results as anti-inflammatory agent, although the mechanisms involved have not been yet completely defined. We thus decided to investigate the effects of GYY-4137 on the nociception and inflammation induced by CGN when injected into the TMJ of rats, and to pharmacologically characterize the mechanisms involved. The experimental protocol was approved by the local Ethics Committee for Animal Experimentation (CEUA-ICB 46, book 2/85, 2010). Under anesthesia with inhalatory isofluorane (3% in O2), male Wistar rats (7 wk. old) received an intra-articular (i.art.) injection of 500 μg of CGN. Four hours later, mechanical allodynia was evaluated by measuring the force threshold necessary for head withdrawal with the aid of an electronic analgesimeter based on the Von Frey filaments principle. Myeloperoxidase (MPO) activity was measured in the TMJ capsule tissue as a marker of neutrophil infiltration. GYY-4137 (1.25-20 μg/joint), glibenclamide (a blocker of ATP-sensitive potassium channels - KATP; 10 and 30 μg/joint), ODQ (1H-(1,2,4) oxadiazolo [4,3-a] quinoxalin-1-one, a specific inhibitor of soluble guanylate cyclase; 0.8 and 8 μg/joint) and sildenafil (a type V-phosphodiesterase inhibitor; 0.3 and 1 μg/joint) were co-injected with CGN. The results were analysed by unpaired Student t-test or ANOVA followed by the

Dunnett's test, when applicable. The i.art. injection of CGN led to an acute TMJ synovitis which was characterized by mechanical allodynia, neutrophil recruitment into the cavity, increased neutrophil infiltrate into the synovial membrane (evaluated as MPO activity) and increased production of the cytokines IL-1β and IL-6. The concomitant administration of GYY-4137 reduced mechanical allodynia and leukocyte influx. Regarding the mechanisms involved, we observed that KATP blockade by glibenclamide antagonized the analgesic effect of GYY-4137 without interfering with leukocyte recruitment. On the other hand, cGMP participates in the anti-inflammatory and antinociceptive actions of H2S in an opposite way, as the inhibition of soluble guanylate cyclase by ODQ antagonized the anti-inflammatory actions of GYY-4137 (but did not affect the antinociceptive effects), and the inhibition of phosphodiesterase-V by sildenafil was able to reverse the antinociceptive action of GYY-4137 without substantially affecting the anti-inflammatory actions (as assessed by tissular MPO activity). The activity of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione transferase) or metalloproteinases (MMP-2 and MMP-9) remained unaltered in response to CGN-induced synovitis or to any of the employed pharmacological treatments. These data provide evidence in favour of the anti-inflammatory and antinociceptive effects of GYY-4137 on the CGN-induced TMJ synovitis in rats, which seem to involve KATP channel activation and cGMP, respectively, thus supporting the therapeutic potential of H2S donors like GYY-4137 to be used in the treatment of temporomandibular disorders of inflammatory etiology.
Financial Support: FAPESP (grant #2014/24518-1), CNPq and CAPES.

References:

1. Ekundi-Valentim et al. Br J Pharmacol. 2010 Apr;159(7):1463-74.

2. Ekundi-Valentim et al. Med Gas Res. 2013 Nov 16;3(1):24. doi: 10.1186/2045-9912-3-2.

Effects of Hypoxia on Intracellular H2S and Polysulfides: Implications in O2 Sensing

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Introduction: The mechanisms by which cells detect hypoxia and transduce this into appropriate physiological responses is a contentious issue. Vascular oxygen sensing is generally attributed to hypoxic generation of reactive oxygen species (ROS). However, H2S and polysulfides (H2Sn) have also been shown to affect redox status (Olson, et. al, 2016). In the past, we have hypothesized that reactive sulfur species (RSS) have the same effect on redox status as ROS and that the metabolism of H2S is the vascular oxygen sensor (Olson, 2014).

Methods: In this study, we examined intracellular production of H2S and H2Sn using 7-Azido-4-Methylcoumarin and 3,6-DI(O-Thiosalicyl (SSP4) respectively in HEK-293 cells and Bovine Pulmonary Artery Smooth Muscle Cells (BPASMC) under both normoxic (21% O2) and hypoxic (0.5% O2) conditions over a 48hr period. In addition we attempted to augment H2S and H2Sn production via their known intracellular production pathways through addition of substrates (to increase production) and inhibitors (to decrease production). Furthermore, we used targeted roGFP transfected cells to the mitochondria and cytosol to examine the redox changes that occur under hypoxic conditions.

Results: H2S production in both BPASMC and HEK-293 cells in normoxic conditions was minimal, whereas in hypoxic conditions, production increased steadily from the 4hr time point, reaching its maximum at 48hr, yielding a two-fold increase in AzMC fluorescence. Polysulfide production showed a steady increase in normoxic conditions, reaching its maximum at 48hr, yielding a two-fold increase in SSP4 fluorescence; however, in hypoxia, SSP4 fluorescence remained constant, showing minimal production. Furthermore, trans-

fected HEK cells showed reduction of the cytosol, but slight oxidation of the mitochondria under hypoxic conditions.

Conclusions: Here, we show an increased production of intracellular H2S under hypoxic conditions (0.5% O2) with a concurrent decrease in intracellular polysulfide production. Additionally, the cytosol of HEK cells becoming further reduced correlates with increased polysulfide production, whereas, oxidation of the mitochondria corresponds to an increased prevalence of polysulfides. Both findings further support the theory that RSS production is directly linked to oxygen concentration, and thereby provide further support for H2S and associated RSS as the oxygen sensor.

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H_2S Restores Carbachol-induced Contractile Responses in Detrusor Smooth Muscle Having Cyclophosphamide-induced Cystitis

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Interstitial cystitis is a syndrome often caused by overactive detrusor and chronic inflammation of bladder and therefore it may change the contractility of the bladder detrusor smooth muscle. In disease situations, muscarinic receptor subtypes in bladder may change (Braverman et al., 1999; Somogyi and de Groat, 1999) and especially an inflammation state may cause detrimental effects on the agonist induced contractile responses (Mok et al., 2000; Palea et al., 1993). The role of endogenous hydrogen sulphide has been demonstrated in human and rat lower urinary tract including bladder smooth muscle (Gai et al., 2013). Since H_2S has also been known to have antioxidant effects, its donors might be useful in preventing the toxic effects of cyclophophamide in bladder such as hemoraggies, contractility changes and functional losses. Therefore, we examined the changes in carbachol-induced contractile responses and the potential therapeutic effect of NaHS in β-escin permeabilized detrusor smooth muscle of mice after cyclophosphamide-induced acute and chronic cystitis. Acute cystitis was induced by cyclophosphamide (CYP, 300 mg/kg, dissolved in saline) that was injected to mice (Swiss, female, 20-25 g) intraperitoneally, with euthanasia occurring 4h after injection. In order to observe whether H_2S prevents the toxic effects of cyclophosphamide, NaHS (10 mg/kg) was injected to mice intraperitoneally before 30 min of CYP injection. Chronic cystitis was induced by CYP (75 mg/kg, dissolved in saline) injected to mice once a day on days 1, 4, 7 and 10 days intraperitoneally. NaHS (10 mg/kg) was injected to treatment group of mice intraperitoneally once every

day (1 hour earlier than injection of CYP) for 10 days. On the eleventh day mice were used for studies. Control groups were injected with saline (%0.9 NaCl). The bladder from mice were isolated and then the mucosa and the connective tissues were removed under a dissecting microscope. Detrusor smooth muscle strips (approximately 150-250 µm in diameter and 3-4 mm in length) were mounted in 1 ml organ baths containing Hepes buffered modified Krebs' solution (NaCl 126; KCl 6; $CaCl_2$ 2; $MgCl_2$ 1.2; glucose 14 and HEPES 10.5 in mM) under a resting tension of 100 mg. Isometric contractions were recorded and expressed as % of 80 mM K^+. Tissues were permeabilized with 100 µM β-escin for 30 min. Carbachol (50 µM)-induced contractions were significantly increased from 18.8±6.1 % (control, n=8) to 44.3±9.3 % in acute cystitis (n=6, $P<0.05$). This elevation in contractile response in acute cystitis group is significantly decreased in NaHS treatment group that is 18.1±6.1 % (n=7, $P<0.05$). Similarly, in chronic cystitis group, carbachol-induced contractions are increased approximately 70 % where NaHS treatment decreased this elevation by roughly 35%. In conclusion, both acute and chronic interstitial cystitis enhances carbachol-induced calcium release. Treatment with the hydrogen sulfide donor NaHS prevents the increase in carbachol-induced contractile responses in both groups. Based on these functional preliminary data, we suggest that H_2S protects the bladder detrusor smooth muscle from the potential detrimental effects of cyclophosphamide.

References:

1. Palea S., Artibani W., Ostardo E., Trist D.G., Pietra C., Evidence for purinergic neurotransmission in human urinary bladder affected by interstitial cystitis, J Urol. 1993;150(6):2007-12.

2. Braverman A., Legos J., Young W., Luthin G., Ruggieri M., M2 receptors in genito-urinary smooth muscle pathology, Life Sci. 1999;64(6-7):429-36.

3. Somogyi G.T., de Groat W.C., Function, signal transduction mechanisms and plasticity of presynaptic muscarinic receptors in the urinary bladder, Life Sci. 1999;64(6-7):411-8.

4. Mok M.H., Knight G.E., Andrews P.L., Hoyle CH., Burnstock G., The effects of cyclophosphamide on neurotransmission in the urinary bladder of Suncus murinus, the house musk shrew, J Auton Nerv System 2000;80(3):30-6.

5. Gai JW., Wahafu W., Guo H., Liu M., Wang X.C., Xiao Y.X., Zhang L., Xin Z.C., Jin J., Further evidence of endogenous hydrogen sulphide as a mediator of relaxation in human and rat bladder, Asian J Androl. 2013;15(5):692-6.

Hydrogen sulfide is a negative regulator of platelet aggregation at phisiologicallt relevant concentrations

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Hydrogen sulfide (H_2S) is generated by a range of human tissues and mediates a variety of biological processes such as vasodilation, inflammation and mitochondrial function (1). The generation of H_2S by platelets is undefined. Functional studies with platelets to date suggest inhibitory roles following exposure to only non-physiological mM concentrations of sulfide salts which release a short burst of H_2S (2). The functional impact of continuous exposure to H_2S at lower concentrations that mimic physiological generation is unknown. In addition, the physiological relevance of endogenous H_2S to platelets remains undefined. We aimed to determine whether and by which enzymes platelets generate H_2S. Secondly, we determined the impact of slow, consistent exposure to H_2S at physiologically relevant concentrations upon platelet function and investigated the role of endogenous H_2S in regulating platelet aggregation *in vivo*. Expression and enzymatic activity of the two principle H_2S-generating enzymes cystathione-γ-lyase (CSE) and cystathionine-β-synthase (CBS) were assessed by western blotting, thialysine (3) and Bindschelder's (4) assays. H_2S generation was measured *via* the H_2S-specific probe WSP-1 and endogenous *S*-sulfhydration measured by a tag-switch assay (5) in human platelet lysates. *In vitro* and *in vivo* platelet aggregation were assessed by light transmission aggregometry of isolated human platelets and in a real-time mouse model of platelet thromboembolism (6). Consistent and robust expression of CBS but

not CSE was detected in human platelets. Platelets also contained CBS but not CSE catalytic activity. H_2S generation by resting platelets could be detected and was significantly reduced upon pharmacological inhibition of CBS. Platelets contained *S*-sulfhydrated proteins.

Exposure of platelets to the slow release donor GYY4137 at concentrations that release H_2S in the nM range led to significant inhibition of aggregation and AP67 which releases H_2S at a greater rate had proportionally higher potency. Pharmacological inhibition of endogenous H_2S generation in mice significantly increased collagen-induced aggregation *in vivo* compared to donor control treated mice. In conclusion, platelets generate H_2S catalytically from CBS which is associated with *S*-sulfhydration of as yet unidentified proteins suggesting novel H_2S-mediated signalling events. Slow consistent exposure to H_2S to mimic physiological release inhibits platelet aggregation in the nM range through mechanisms that remain to be identified. Finally, endogenous H_2S is a negative systemic regulator of platelet aggregation and may be hypothesised to exert anti-thrombotic activity *in vivo*.

References:

1. Whiteman M., Le Trionnaire S., Chopra M., Fox B., Whatmore J. Emerging role of hydrogen sulfide in health and disease: critical appraisal of biomarkers and pharmacological tools. Clin Sci (Lond).121(11):459-88.

2. Zagli G., Patacchini R., Trevisani M., Abbate R., Cinotti S., Gensini G.F., et al. Hydrogen sulfide inhibits human platelet aggregation. European journal of pharmacology. 2007;559(1):65-8.

3. Ogasawara Y., Ishii K., Tanabe S. Enzymatic assay of gamma-cystathionase activity using pyruvate oxidase-peroxidase sequential reaction. Journal of biochemical and biophysical methods. 2002;51(2):139-50.

4. Shen W., McGath M.K., Evande R., Berkowitz D.B. A continuous spectrophotometric assay for human cystathionine beta-synthase. Analytical biochemistry. 2005;342(1):103-10.

5. Zhang D., Macinkovic I., Devarie-Baez N.O., Pan J., Park C.M., Carroll K.S. et al. Detection of protein S-sulfhydration by a tag-switch technique. Angew Chem Int Ed Engl. 2014;53(2):575-81.

6. Tymvios C., Jones S., Moore C., Pitchford S.C., Page C.P., Emerson M. Real-time measurement of non-lethal platelet thromboembolic responses in the anaesthetized mouse. Thrombosis and haemostasis. 2008;99:435-40.

Pro-apoptotic effect of H_2S-donors on human melanoma cells

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Cutaneous malignant melanoma is the most aggressive form of skin cancer, with a high mortality rate. Various treatments for malignant melanoma are available, but due to the development of multi-drug resistance, current or emerging chemotherapies have a relatively low success rates. This emphasizes the importance of discovering new compounds that are both safe and effective against melanoma (Chinembiri et al., 2014). In the last few years, numerous physiological and pathophysiological roles have been proposed for the gasotransmitter hydrogen sulphide (H_2S) along with a plethora of cellular and molecular targets. H_2S is endogenously produced by the action of three enzymes CBS, CSE and the newly discovered 3-MST (Wang R. et al., 2012). While H_2S is cytoprotective at physiological concentrations, it seems to have pro-apoptotic actions in cancer cells (Predmore BL. et al., 2012). However, to date there are not definitive reports on the role played by H_2S in cancer development. To gain further insights into the role played by H_2S in human melanoma, we evaluated the effect of several H_2S-donors (NaHS, DATS, GYY4137, Thioglicyne and Thiovaline), on A375 melanoma cell proliferation. All the compounds, but not NaHS, inhibited the growth of A375 cells in a concentration-dependent manner. Among all the H_2S-donors tested we focused our attention on the potential antitumor effect of synthetic H_2S-donor GYY4137. The effects of this compound was also evaluated on other three different human melanoma cell lines: SK-Mel-5, SK-Mel-28 and PES43. We found that GYY4137 decreased in a concentration-dependent manner the proliferation rate

of all melanoma cells used. Normal human epidermal melanocytes (NHEM) were used as control. In order to evaluate if the anti-proliferative effects of GYY4137 was due to apoptosis or necrosis, flow cytometry analysis by double staining with Annexin V and propidium iodide (PI) was carried out. This dual staining distinguishes between unaffected cells, early apoptotic cells, late apoptotic cells and necrotic cells. Treatment of A375 cells for 24, 48 and 72h with GYY4137 (1000µM) resulted in a time-dependent induction of apoptosis. In particular, at 72h almost 40% cells exhibited markers of late apoptosis. This effect was accompanied by a time-dependent activation of caspase 3 and the cleavage of its substrate poly(adenosine diphosphate-ribose) polymerase (PARP). The main transcription factor involved in the regulation of apoptosis is NF-κB, thus we hypothesized that the H_2S donors induction of apoptosis was associated with suppression of NF-κB activation (Wang CY. et al., 1999). Western blot analysis carried out on the nuclear extracts of A375 cells incubated with GYY4137 for 3-6-24h resulted in a time-dependent reduction of p65 nuclear translocation and activation. Moreover, the expression of the anti-apoptotic proteins c-FLIP, XIAP and Bcl-2, that is transcriptionally regulated by NF-κB (Ben-Neriah and Karin, 2011), was greatly reduced following treatment of cells with GYY4137 1000 µM for 3-6 and 24h. Two of the most frequently deregulated pathways in melanoma are mitogen-activated protein kinase (MAPK)/ERK and phosphoinositide 3-kinase (PI3K)/AKT (Hodis et al., 2012). These two pathways play an important role in melanoma development and progression and are involved in the mechanism of resistance to targeted therapy (Flaherty et al., 2012). Western blot analysis revealed that treatment of A375 cells with GYY4137 1000µM inhibited the phosphorylation and activation of both AKT and ERK at all time considered (3-6-24h).

In conclusion we have demonstrated that both natural and synthetic H_2S-donors inhibits human melanoma cells proliferation by inhibiting pro-survival pathways associated to NF-κB activation. Our results establish H_2S-donors as new potential agents in the treatment of human metastatic melanoma and represent a very promising strategy to improve the fight against cancer.

References:

1. Wang C.Y., Guttridge D.C., Mayo M.W., Baldwin AS Jr. NF-kappaB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. Mol Cell Biol. 1999 Sep;19(9):5923-9.

2. Ben-Neriah Y., Karin M. Inflammation meets cancer, with NF-κB as the matchmaker. Nat Immunol. 2011 Jul 19;12(8):715-23.

3. Wang R. Physiological implications of hydrogen sulfide: a whiff exploration that blossomed. Physiol Rev. 2012 Apr;92(2):791-896.

4. Predmore B.L., Lefer D.J., Gojon G. Hydrogen sulfide in biochemistry and medicine. Antioxid Redox Signal. 2012 Jul 1;17(1):119-40

5. Hodis E., Watson I.R., Kryukov G.V., Arold S.T., Imielinski M., Theurillat J.P., Nickerson E., Auclair D., Li L., Place C., Dicara D., Ramos A.H. et all. A landscape of driver mutations in melanoma. Cell. 2012 Jul 20;150(2):251-63.

6. Flaherty K.T., Infante J.R., Daud A., Gonzalez R., Kefford R.F., Sosman J., Hamid O., Schuchter L., Cebon J., Ibrahim N., Kudchadkar R., Burris H.A. 3rd, Falchook G., Algazi A., Lewis K., Long G.V., Puzanov I., Lebowitz P., Singh A., Little S., Sun P., Allred A., Ouellet D., Kim K.B., Patel K., Weber J. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. N Engl J Med. 2012 Nov;367(18):1694-703.

7. Chinembiri T.N., du Plessis L.H., Gerber M., Hamman J.H., du Plessis J. Review of natural compounds for potential skin cancer treatment. Molecules. 2014 Aug6;19(8):11679-721.

Effects of 7 days melatonin introduction on the hydrogen sulfide and glutathione level in the blood of diabetic rats with nephropathy

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Diabetes mellitus and its complications represent a major sociomedical problem today. Hyperglycemia is a driving force for the development of diabetic nephropathy [5]. Changes in the concentration and production of hydrogen sulfide play an important role in the pathogenesis of pancreas β-cell dysfunction and in the pathogenesis of endothelial injury, which develop on the basis of elevated circulating glucose levels in diabetes mellitus [4]. The experiment was carried out on male albino rats with the body weight of 0.16 – 0.18 kg. Experimental diabetes was induced by 5% alloxan monohydrate solution in the dose of 150 mg/kg. Nephropathy was induced in diabetic rats by injection of glycerol (10 mg/kg) after fasting overnight. After diabetes and nephropathy were confirmed, rats were divided into five groups: 1) control rats; 2) alloxan diabetic rats; 3) animals with overt diabetes, which introduced to melatonin intragastrically in the dose of 10 mg/kg at 8 a.m. during 7 days; 4) animals with diabetic nephropathy; 5) animals with diabetic nephropathy, introduced to melatonin intragastrically in the dose of 10 mg/kg at 8 a.m. during 7 days. We determined concentration of H_2S and glutathione in the blood, activity of glutathione-S-transferase (G-S-T) and glutathione peroxidase (GPx) in the blood, activity of cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) in the liver. Fasting blood glucose level was increased in diabetic rats in 2.3 times, but the introduction of melatonin promoted normalization of the level of basal glycaemia in diabetic animals, indicating hypoglycemic action of melatonin. In rats with diabetic nephropathy the level of glucose increased in 1.9 times as compared with the level of glucose in control rats. Melatonin does not show hypoglycemic action in combined pathology. Our study shows the plasma H_2S level is

significantly reduced in the alloxan induced diabetic rats in 1.3 times and in rats with nephropathy in 1.8 times as compared with plasma level of H_2S in control rats. In the blood of alloxan diabetic rats receiving melatonin the content of H_2S increases as compared with the groups of diabetic rats and rats with nephropathy in 1.2 and 1.6 times accordingly. Our examination demonstrates a reduction of CSE activity in the liver of alloxan-diabetic rats in 1.5 times and in 2.6 times there was reduction in rats with diabetic nephropathy. Similar changes have been observed in activity of CBS in the liver of alloxan-diabetic rats and rats with combination pathology. The introduction of melatonin contributes to the increasing CSE activity in the groups of diabetic rats and activity of CBS in rats with combined pathology. Hyperglycemia leads to depletion of the antioxidant defense mechanism, thus promotes the generation of free radicals resulting in an endothelial dysfunction [3]. Metabolism of glutathione is known to be associated with the metabolism of sulfur-containing aminoacids and hydrogen sulfide. The glutathione system is one of the most powerful antioxidant cell systems, interaction of free radicals, and protects against lipid peroxidation [1, 2]. Our results indicate the level of glutathione in red blood is reduced as compared with the level in control rats (in 1.3 times in the alloxan induced diabetic rats and in 1.2 times in diabetic rats with nephropathy). The activity of G-S-T and GPx increased in red blood cells in 1.25 times in the alloxan induced diabetic rats, and in 1.2 times in diabetic rats with nephropathy. The introduction of melatonin contributes to the normalization an activity of G-S-T in the alloxan induced diabetic rats and in rats with nephropathy, and the activity of GPx normalizes only in group of animals with diabetes mellitus. It may indicate the protective functions against oxidative stress. Study of H_2S concentration and its producing enzymes may prove to be an effective strategy for modulating diabetes treatment. The disorders of metabolism of hydrogen sulfide in alloxan diabetic rats can lead to the imbalance between oxidative and reductive species. Our results suggest that melatonin is effective for the normalization of metabolism of hydrogen sulfide and glutathione system in diabetic rats. The introduction of melatonin in the group of diabetic rats with nephropathy didn’t show reliable changes, and therefore needs further studies using melatonin for a longer period of

time.

References:

1. Townsend D.M., Tew K.D. The importance of glutathione in human disease. Biomed. Pharmacother. 2003;57:145–155.

2. Yonei Y., Hattori A., Tsutsui K., Okawa M., Ishizuka B. Effects of Melatonin: Basics Studies and Clinical Applications. Anti-Aging Medicine. 2010;7:85–91.

3. Shen X. Pattillo CB. Pardue S. Bir SC. Wang R. Kevil C.G. Measurement of plasma hydrogen sulfide in vivo and in vitro. Free Radic Biol Med. 2011;50:1021–1031.

4. Okamoto M., Ishizaki T., Kimura T. Protective effect of hydrogen sulfide on pancreatic beta-cells. Nitric Oxide. 2013;46:227 – 233.

5. Sun Y.M., Su Y., Li J. Recent advances in understanding the biochemical and molecular mechanism of diabetic nephropathy. Biochem. Biophys. Res. Commun. 2013;433:359 –361.

Thiosulfate sulfur-transferase gene deficiency activates aortic nitric oxide signaling and ameloriates vascular function after a diabetogenic challenge

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Thiosulfate sulfurtransferase (*Tst*, aka Rhodanese) is indirectly linked to breakdown of the gasotransmitter hydrogen sulfide (H_2S) (2). H_2S is emerging as a key gasotransmitter involved in vasodilation of the vascular system (3) and has been shown to protect endothelial cells from the nutrient dysregulation seen in diabetes (1). It was hypothesised that genetic deletion of *Tst* ($Tst^{-/-}$ mice) would lead to altered aortic vasodilation and protection of endothelial function in a mouse diabetic model. This investigation aimed to determine whether *Tst* is expressed in the aorta, and whether its deletion alters the endothelial nitric oxide synthase (eNOS) signalling pathway in control and diabetic animals. *Tst* mRNA was detected in the aortic wall. Phosphorylation of eNOS protein at the activating Ser1177 site was greater in aortas from $Tst^{-/-}$ mice compared with wild type ($Tst^{+/+}$) mice. Vascular responses in aorta from control animals to the vasoconstrictors phenylephrine (PE) and 5-hydroxytryptamine (5-HT), and the vasodilator acetylcholine (ACh) were comparable in $Tst^{-/-}$ and $Tst^{+/+}$. Inhibition of eNOS and cyclooxygenase (COX) had similar effects in both genotypes. Other than a small decrease in the sensitivity of vessels to 5-HT (< 0.15 decrease in pEC_{50} value, 2 way ANOVA diet $p = 0.047$) high fat diet (HFD) feeding for 7 or 20 weeks did not alter functional responses in aorta from $Tst^{+/+}$ or $Tst^{-/-}$ mice. However, when eNOS and COX were inhibited, an unexpected increase in residual vasodilation was observed in $Tst^{-/-}$ but not $Tst^{+/+}$ mice (7 weeks; maximal relaxation; $Tst^{+/+}$ 19.5 ± 3.8%, $Tst^{-/-}$: 41.3 ± 3.8%, Student's t test $p = 0.007$). Further, after 20 weeks HFD

maximal constriction of *Tst*$^{+/+}$ vessels to PE was decreased (20 weeks; maximal constriction; Control: 240.8 ± 8.1%, HFD: 187.2 ± 22.9%) under combined eNOS and COX inhibition. Strikingly, this did not occur in *Tst*$^{-/-}$ mice where responses were identical (20 weeks; maximal constriction; Control: 253.0 ± 12.3%, HFD: 267.3 ± 15.7%, Bonferroni post hoc test *Tst*$^{+/+}$ HFD vs. *Tst*$^{-/-}$ HFD $p < 0.01$). In conclusion, this work has shown that *Tst* is present in the vessel wall and deletion of *Tst* increases activating phosphorylation of eNOS in the endothelium. Despite this investigations showed that there were no apparent changes in baseline vascular function of control or HFD fed mice. However, pharmacological inhibition of endothelium-derived relaxing factors (NO, prostaglandins) in HFD fed mice did reveal alterations in the contribution of vasodilators in *Tst*$^{-/-}$. It also uncovered that maximal constriction was decreased in *Tst*$^{+/+}$ mice fed HFD but appeared to be protected in *Tst*$^{-/-}$. These findings suggest that *Tst* interacts with the endothelial NO signalling system but require further investigation.

References:

1. Suzuki K, Olah G, Modis K, Coletta C, Kulp G, Gerö D, et al. Hydrogen sulfide replacement therapy protects the vascular endothelium in hyperglycemia by preserving mitochondrial function. Proc Natl Acad Sci U S A. 2011 Aug 16;108(33):13829–34. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3158211&tool=pmcentrez&rendertype=abstract

2. Libiad M, Yadav PK, Vitvitsky V, Martinov M, Banerjee R. Organization of the human mitochondrial H2S oxidation pathway. J Biol Chem. 2014 Sep 15;289(45):30901-10 Available from: http://www.jbc.org/cgi/doi/10.1074/jbc.M114.602664

3. Wang R, Szabo C, Ichinose F, Ahmed A, Whiteman M, Papapetropoulos A. The role of H2S bioavailability in endothelial dysfunction. Trends Pharmacol Sci. Elsevier Ltd; 2015 Jun 09;36(9):568-78. Available from: http://linkinghub.elsevier.com/retrieve/pii/S016561471500098

Thiosulfate sulfur-transferase deficiency engenders a diabetogenic metabolic profile in hepatocytes

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Thiosulfate sulfurtransferase (*Tst*, aka rhodanese) is a nuclear-encoded mitochondrial protein indirectly linked to the breakdown of the gasotransmitter H_2S (3). *Tst* is highly expressed in the liver, where H_2S signalling regulates metabolism (2). Elevated hepatic H_2S has been linked with decreased insulin sensitivity and increased glucose production, hallmarks of diabetes (1). However, the role of enzymatic H_2S breakdown in hepatic H_2S exposure is unknown. We hypothesised that deletion of the *Tst* gene attenuates hepatic H_2S breakdown and contributes to the development of type 2 diabetes. To test this we investigated H_2S and energy metabolism in $Tst^{-/-}$ mice before performing proteomics analysis of whole liver and mitochondria in $Tst^{-/-}$ and $Tst^{+/+}$ to gain insight into the molecular pathways underpinning any effects. Consistent with the canonical role ascribed to TST, thiosulfate levels were elevated in plasma and urine of $Tst^{-/-}$ mice ($Tst^{-/-}$ 66 ± 9.1 µM, $Tst^{+/+}$ 2.8 ± 0.12 µM, $p < 0.0001$). Monobromobimane (MBB) derivatisation also revealed markedly increased H_2S in whole blood of $Tst^{-/-}$ mice ($Tst^{-/-}$ 227 ± 9.2 µM, $Tst^{+/+}$ 30.3 ± 1.3 µM, $p < 0.001$). Hepatic glucose production was higher in $Tst^{-/-}$ mice after intraperitoneal pyruvate challenge (AUC glucose; $Tst^{-/-}$ 1239 ± 71.8, $Tst^{+/+}$ 1061 ± 36.3, $p = 0.037$). In support of elevated hepatic glucose production, phosphoenolpyruvate carboxykinase (PEPCK) activity was increased in $Tst^{-/-}$ mice (NADH extinction measured at 340 nM; $Tst^{-/-}$ -0.1 ± 0.01 signal/min/g protein, $Tst^{+/+}$ -0.076 ± 0.01 signal/min/g protein, $p = 0.0495$). $Tst^{-/-}$ mice also exhibited increased plasma VLDL triglyceride levels ($Tst^{-/-}$ 45.6 ± 6.6

mg/dL, *Tst*$^{+/+}$ 26 mg/dL ± 4.5, p = 0.034) reminiscent of a diabetic liver lipid export profile. Primary hepatocytes from *Tst*$^{-/-}$ mice exhibited an increased rate of oxygen consumption (OCR, *Tst*$^{-/-}$ 113.9 ± 7.7 pmol/min/protein, *Tst*$^{+/+}$ 83.9 ± 10.5 pmol/min/protein, p = 0.01) that comprised of increased ATP-coupled (*Tst*$^{-/-}$ 76.1 ± 5.1 pmol/min/protein, *Tst*$^{+/+}$ 59.9 ± 7.2 pmol/min/protein, p = 0.032) and non ATP-coupled leak (*Tst*$^{-/-}$ 37.7 ± 3.3 pmol/min/protein, *Tst*$^{+/+}$ 23.9 ± 4.2 pmol/min/protein, p = 0.005) respiration. Notably, isolated liver mitochondria from *Tst*$^{-/-}$ mice unexpectedly showed higher rates of H_2S breakdown (*Tst*$^{-/-}$ 1220 ± 152.1 pmol/min/mg protein, *Tst*$^{+/+}$ 710 ± 87.6 pmol/min/mg protein, p = 0.03), suggesting a compensatory up-regulation of alternative pathways for H_2S detoxification. Quantitative proteomics identified altered levels for proteins in key pathways including decreased pyruvate dehydrogenase kinase 1 (PDK1, pyruvate entry to mitochondrial TCA cycle), increased low density lipoprotein receptor-related protein 1 (LRP1, lipid uptake/transport), increased betaine homocysteine methyl transferase 1 (BHMT1, the transulfuration pathway) and increased heme oxygenase 1 (HO-1, heme breakdown/antioxidant pathway) that begin to provide a molecular framework for altered metabolism in hepatocytes of *Tst*$^{-/-}$ mice.

Our data suggest that *Tst* gene deletion invokes multiple changes in hepatocyte glucose and lipid metabolism that ultimately confer a susceptibility to diabetes. Moreover, despite a compensatory increase in H_2S breakdown at the hepatocellular level, higher sulfide exposure may negatively dominate hepatic function in *Tst*$^{-/-}$ mice.

References:

1. Zhang L., Yang G., Untereiner A., Ju Y., Wu L., Wang R. Hydrogen sulfide impairs glucose utilization and increases gluconeogenesis in hepatocytes. Endocrinology. 2013 Jan;154(1):114–26. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23183179

2. Mani S., Cao W., Wu L., Wang R. Hydrogen sulfide and the liver. Nitric Oxide. Elsevier Inc. 2014 Feb 26;41:62-71 Available from: http://www.ncbi.nlm.nih.gov/pubmed/24582857

3. Libiad M, Yadav PK, Vitvitsky V, Martinov M, Banerjee R. Organization of the human mitochondrial H2S oxidation pathway. J Biol Chem. 2014 Sep 15;289(45):30901-10 Available from: http://www.jbc.org/cgi/doi/10.1074/jbc.M114.602664

The Role of Hydrogen Sulfide and Inflammation in Experimental Autoimmune Diabetes

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Type 1 diabetes is an autoimmune disease caused by β-cell destruction in the pancreas. Studies have suggested a potential role for H_2S in the development of type 1 diabetes, with inhibition of cystathionine gamma-lyase (CSE) offering protection from streptozotocin (STZ) induced β-cell death in vitro (3) and delaying the onset of hyperglycemia and protecting β-cells in vivo (5).

Others have shown induction of cystathionine beta synthase (CBS) in pancreas of STZ treated rats in vivo (1). Conversely, H_2S donors have been shown to inhibit leukocyte adherence, and inhibition of endogenous H_2S production promotes leukocyte adherence (2), which may influence leukocyte recruitment during type 1 diabetes. Furthermore, Non-obese diabetic (NOD) mice, which spontaneously develop Type 1 diabetes, have been shown to have progressively reduced plasma H_2S levels (4). However, the mechanisms by which hydrogen sulfide contributes to or protects from the pathogenesis of type 1 diabetes are not clear. Here we examine the relationship between CBS, CSE, and 3-MST expression and levels of endothelial cell adhesion molecules involved in leukocyte adhesion required for insulitis. Pancreatic endothelial cells were isolated from NOD, ALR, and IDD22 mice, as well as the aorta of NOD mice, which acted as a control. Protein levels of CBS, CSE, and 3-mercaptopyruvate sulfurtransferase (3-MST) were measured by western blot from endothelial cell lysates. CSE protein expression was lower in all pancreatic endothelial cells compared to aortic endothelial cells. Pancreatic endothelial cells isolated from the ALR mice showed an absence of CSE expression and an increase in 3-MST expression compared to NOD and IDD22 mice. IDD22 mice showed a decrease in CBS expression compared to NOD and ALR mice. Flow cytometry

data indicate that endothelial cells isolated from NOD mouse pancreas have significantly elevated levels of E-Selectin, P-Selectin, ICAM-1 and VCAM-1 compared to ALR and IDD22 mice as well as NOD aortic endothelial cells both basally and following 4 hours stimulation with 10 ng/ml TNFα. Selectin levels of IDD22 and ALR pancreatic endothelial cells were similar, whereas ICAM-1 and VCAM-1 were elevated in IDD22 mice over ALR mice following 4 hours TNFα stimulation. These differences in expression of CBS, CSE and 3-MST may contribute to susceptibility to or protection from the development of type 1 diabetes.

References:

1. Yusuf M., Kwong Huat B.T., Hsu A., Whiteman M., Bhatia M., and Moore P.K. Streptozotocin-induced diabetes in the rat is associated with enhanced tissue hydrogen sulfide biosynthesis. Biochem Biophys Res Commun. 2005;333:1146-1152.

2. Zanardo R.C., Brancaleone V., Distrutti E., Fiorucci S., Citrino G. and Wallace J.L. Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. FASEB J. 2006;20: 2118-2120.

3. Yang G., Yang W., Wu L. and Wang R. H_2S, endoplasmic reticulum stress, and apoptosis of insulin-secreting β cells. J Biol Chem. 2007;282: 16567-16576.

4. Brancaleone V., Roviezzo F., Vellecco V., De Gruttola L., Bucci M. and Citrno G. Biosythesis of H2S is impaired in non-obese diabetic (NOD) mice. Br J Pharmacol. 2008;155:673-680.

5. Yang G., Tang G., Zhang L., Wu L., and Wang R. The pathogenic role of cystathionine γ-lyase/hydrogen sulfide in streptozotocin-induced diabetes in mice. Am J Pathol. 2011;179:869-879.

Hydrogen sulfide supplementation mitigates ischemia reperfusion injury-associated renal graft injury in a murine model of donation after cardiac death renal transplantation

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Current strategies to expand the supply of available donor organs for transplantation has included greater use of donation after cardiac death (DCD) organs. However, DCD renal grafts experience prolonged periods of warm ischemia in addition to cold ischemia reperfusion injury (IRI) that results in higher rates of delayed graft function and failure compared to standard criteria donor organs (1). Our lab has previously shown that hydrogen sulfide (H2S) supplementation mitigates renal injury in models of both warm and cold IRI (2-5). This study aims to investigate if H2S supplementation in a model of DCD renal transplantation (RTx) combining warm and cold ischemia can reduce renal graft injury and improve recipient outcomes. Adult male Lewis rats either underwent a midline incision only (Sham group; n=5) or received a renal transplant from a syngeneic donor following bilateral nephrectomy (UW and H2S groups; n=6 per group). Donor kidneys were subjected to 30 minutes of warm (37 ºC) ischemia via clamping of renal pedicle followed by 18 hours of cold storage (4 ºC) in standard University of Wisconsin (UW) organ preservation solution prior to transplantation. Donor rats in the H2S treated group were injected intra-peritoneally with D-cysteine (2mmol/kg) 1 hour before procurement and the UW solution was supplemented with 150μM NaHS during cold storage. Donor rats in the UW group received only PBS injections prior to procurement and kidneys were stored in UW solution alone during cold storage. Transplant recipients were monitored for 30 days or until the time of sacrifice. Blood and urine samples were col-

lected for analysis of renal function and the graft was recovered for histopathology at the time of sacrifice or death. H2S supplementation provided survival benefit as exhibited by significantly improved survival of the H2S treated group in comparison to untreated group ($p<0.05$). H2S treated animals also exhibited improved renal graft function as evident from decreased urine protein to urine creatinine ratio ($p<0.05$ for day 5) and the trend of decreased serum creatinine compared to UW treated animals. Further, while UW grafts exhibited significantly greater acute tubular necrosis (ATN) scores ($p<0.05$) compared to Sham, ATN scores in H2S treated grafts were not significantly different compared to Sham. This is the first study depicting the benefits of H2S supplementation in a clinically relevant murine model of DCD renal transplantation. These findings could potentially represent a novel method of reducing both warm and cold IRI during DCD transplantation and ultimately improving recipient outcomes for this rapidly growing subset of donor organs.

References:

1. Snoeijs M.G., Winkens B., Heemskerk M.B. et al: Kidney transplantation from donors after cardiac death: a 25-year experience. Transplantation. 2010;10:1106.

2. Zhu J.X., Kalbfleisch M., Yang Y.X. et al: Detrimental effects of prolonged warm renal ischaemia-reperfusion injury are abrogated by supplemental hydrogen sulphide: an analysis using real-time intravital microscopy and polymerase chain reaction. BJU Int. 2012;(11):E1218.

3. Lobb I., Zhu J., Liu W. et al: Hydrogen sulfide treatment ameliorates long-term renal dysfunction resulting from prolonged warm renal ischemia-reperfusion injury. Can Urol Assoc J. 2014;8:E413.

4. Lobb I., Mok A., Lan Z. et al: Supplemental hydrogen sulphide protects transplant kidney function and prolongs recipient survival after prolonged cold ischaemia-reperfusion injury by mitigating renal graft apoptosis and inflammation. BJU Int. 2012;(11):E1187.

5. Lobb I., Davison M., Carter D. et al: Hydrogen sulfide treatment mitigates renal allograft ischemia reperfusion injury during cold storage and improves early transplant kidney function and survival following allogeneic renal transplantation. J Urol. 2015 Dec;(6):1806.

Polysulfides as Intermediate Species and Products of Nitrosopersulfide Synthesis and Decomposition

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The "cross-talk" between H_2S and NO signaling was demonstrated in a number of recent studies (reviewed in [1]). As a starting point to dissect the underlying chemical foundations of the cooperative interactions of NO and H_2S, here we investigated the reaction of the model S-nitrosothiol (S-nitroso-N-acetyl-D,L-penicillamine; SNAP) with H_2S. The reaction leads to the generation of a number of sulfane sulfur containing and S/N hybrid inorganic molecules, with major characterized products at an excess of sulfide being: polysulfides (HS_x^-), nitrosopersulfide ($SSNO^-$), dinitrososulfite (SULFI/NO) and nitroxyl (HNO) [2]. We obtained evidence -on kinetic grounds as well as by chemical characterization -for polysulfide generation during the early phase of the reaction. Polysulfides were found to be more reactive with SNAP than sulfide itself and served as autocatalytic intermediate species in the overall reaction to give the other major product, $SSNO^-$. Autocatalytic formation of $SSNO^-$ was apparent based on the observed induction periods in the kinetic traces at λ = 412 nm (the absorption maximum of $SSNO^-$). Addition of preformed polysulfides to reagent sulfide solutions diminished this induction period (which disappeared at sufficient polysulfide concentrations), corroborating that polysulfides, that were generated in situ in the reactions of SNAP with H_2S, were indeed the catalyzing intermediate species of $SSNO^-$ formation. $SSNO^-$ decomposition is a relatively slow process accompanied by the release of NO, where the oxidizing

equivalents are eventually mostly converted to polysufide formation. Quantitative measurements of total sulfur content in $SSNO^-$ (assessed by sulfide quantification upon the reduction of sulfane sulfur species during $SSNO^-$ decomposition in a time resolved manner) correlated well with the amount of released sulfane sulfur (measured by cold cyanolysis and extraction of polysulfide sulfane sulfurs into chlorophorm). These quantitative analyses indicated that $SSNO^-$ accounts for 25-30% of the oxidizing equivalents coming from SNAP.
In conclusion, our results suggest that nitrosopersulfide, which is a major product of chemical interactions between NO and H_2S, can serve as a reservoir and donor of sulfane sulfur and nitric oxide and hence could play a role in sustained NO and sulfide signaling.

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References:

1. Kevil C., Cortese-Krott M.M., Nagy P., Papapetroupulos A., Feelisch M. and Szabo C. Cooperative interactions between NO and H_2S: chemistry, biology, physiology, pathophysiology Nitric Oxide Biology and Pathobiology Ignarro L.J. Ed, third edition, in press.

2. Cortese-Krott M.M., Kuhnle G.G., Dyson A., Fernandez B.O., Grman M., DuMond J.F., Barrow M.P., McLeod G., Nakagawa H., Ondrias K., Nagy P., King S.B., Saavedra J.E., Keefer L.K.,Singer M., Kelm M., Butler A.R., Feelisch M. Key bioactive reaction products of the NO/H_2S interaction are S/N-hybrid species, polysulfides, and nitroxyl. Proc Natl Acad Sci U S A. 2015 Aug 25;112(34):E4651-60.

Different Contribution of Nitric Oxide and Hydrogen Sulfide to Endothelium-Dependent Vasodilatation in Rat Mesenteric Arteries

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Recently we have found that hydrogen sulfide (H_2S) causes vasodilation in rat mesenteric arteries by opening of voltage-gated K channels followed by lowering of smooth muscle cell calcium as well as by desensitization by a mitochondrial mechanism involving complex I and III (Hedegaard et al., J Pharmacol Exp Ther., 2016). In the present study we investigated whether H_2S contributes to acetylcholine relaxation in rat mesenteric arteries. Rat mesenteric arteries were mounted in microvascular myographs for pharmacological studies and for simultaneous measurements of relaxation and nitric oxide (NO) or H_2S by use of amperometric microsensors for these gases. In norepinephrine-contracted arteries, acetylcholine induced endothelium-dependent relaxations and increases in nanomolar NO concentrations which were inhibited in the presence of inhibitors of NO synthase, nitro-L-arginine (L-NOARG) and asymmetric dimethyl-arginine (ADMA). H_2S relaxations were unaltered after endothelial cell removal, and in the presence of NO synthase inhibitors and indomethacin. The endogenous H_2S production was blocked with the enzyme inhibitors of, respectively, cystathione-γ-lyase and cystathione-β-synthetase, dl-propargylglycine (PPG) and amino-oxyacetate (AOA), which inhibited acetylcholine relaxation in the absence, but not in the presence of L-NOARG, while relaxations induced by an NO donor, sodium nitroprusside were unaltered. Simultaneous measurements of relaxation and free H_2S concentration revealed that the exogenously-added H_2S salt, NaHS, caused concentration-dependent relaxations. Thus, at 300 µM NaHS, the H_2S concentration measured with intraluminal and extraluminal H_2S microsensors was, respectively, 15.6 µM and 6.5 µM, and the

artery relaxed ~62%. Acetylcholine failed to increase the H_2S concentration. Blockers of voltage-gated K_V7 channels inhibited NaHS relaxation, and blockers of mitochondrial complex I and III abolished NaHS relaxation. Our findings suggest that in rat mesenteric arteries, endothelium-derived NO and hyperpolarization contribute to acetylcholine relaxation, while H_2S at micromolar may modulate these relaxations at mitochondrial level.

References:

1. Hedegaard E.R., Johnsen J., Povlsen J.A., Jespersen N.R., Shanumaganathan J.A., Kristiansen S.B, Simonsen U., Bøtker H.E. Inhibition of K_V7 channels provides coronary flow dependent protects against myocardial ischemia and reperfusion injury. Journal of Pharmacology and Experimental Therapeutics. 2016 Feb 11;pii: jpet.115.230409.

H_2S-Induced er stress in A2870 cells is increased under hypoxia

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Hypoxia - a state of lower oxygen demand - is responsible for a higher aggressiveness of tumors and therefore a worse prognosis. During hypoxia, several metabolic pathways are re-organized, e.g. energetic metabolism, modulation of pH, calcium transport. Calcium is an important second messenger that regulates variety of processes in the cell. Thus, aim of this work was to compare H_2S modulation of the intracellular calcium transport systems in hypoxia and in cells grown in standard culture conditions. For all experiments we used ovarian cancer cell line (A2780). H_2S is a novel gasotransmitter, known to be involved in a modulation of several calcium transport systems, thus resulting in altered calcium signaling. Two models of hypoxia were used in our study – chemical (induced by dimethyloxallyl glycine) and 2% O_2 hypoxia, both combined with a treatment using a slow H_2S donor GYY4137. In hypoxia, we observed rapid changes in cytosolic and reticular calcium levels compared to cells grown in standard culture conditions and these changes were even more exagerrated when combined with the GYY4137. Changes in a calcium homeostasis result from IP_3 receptor´s upregulation and downregulation of the SERCA 2, which leads to a development of the endoplasmic reticulum stress. Based on our results we propose a higher vulnerability of calcium transport systems to H_2S regulation under hypoxia.

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A Novel Quantitative Analysis of Polysulfur by Modified Methylene Blue Assay

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Sulfide Anti-Oxidant Buffer (SAOB), composed of 0.5 M sodium salicylate, 0.12 M ascorbic acid and 2.2 M NaOH, was usually used for ion selective electrodes (ISE) to avoid H_2S oxidation in 1960s-1970s. Interestingly, it was previously reported that plasma H_2S produced by SAOB reached to millimolar level.[1]. In addition, Olson KR interpreted that these H_2S most likely came from thiol by the reaction as follows; $RSH + 2OH \rightarrow ROH + S^{2-} + H_2O$ [2]. Therefore, there is an ongoing debate about this origin of H_2S induced by SAOB method yet. On the other hand, it was suggested that some protein like albumin has polysulfur in its cysteine [3] and polysulfur might possibly produce H_2S with SAOB. In this study, we examined the relationship between SAOB and thiol/polysulfur in human serum samples. First, we detected H_2S in human serum at millimolar level by methylene blue assay using SAOB (MB-S). To clarify the origin of H_2S induced by SAOB, we purified human serum albumin (HSA) from human serum, and then measured the amount of H_2S in the purified HSA by MB-S. As the result, H_2S was produced from the HSA more than about 10 mol/mol HSA. We also confirmed that a hydroxyl group was not induced from a thiol group by SAOB reaction. Furthermore, some sulfane sulfur was also detected from the purified HSA by SSP4 probe, which is one of the sulfane sulfur probes

synthesized by Dr. Ming. These data suggest that endogenous HSA play an important role as a reservoir of sulfane sulfur in human serum. In conclusion, MB-S could be a novel quantitative analysis to detect the endogenous polysulfur or bound sulfur species.

References:

1. Whitfield N.L., Nathan L. et al. "Reappraisal of H2S/sulfide concentration in vertebrate blood and its potential significance in ischemic preconditioning and vascular signaling." American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2008;294:6 R1930-R1937.

2. Olson K.R., Kenneth R., Eric R. DeLeon, and Fang Liu. "Controversies and conundrums in hydrogen sulfide biology." Nitric oxide. 2014;41:11-26.

3. Westley, John et al. "The sulfurtransferases." Toxicological Sciences. 1983;3.5:377-382.

The Role of Reverse Transsulfuration Enzymes in Ulcerative Colitis and Colitis-Associated Carcinogenesis

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Objectives/Aims: Ulcerative colitis (UC) is a lifelong inflammatory disease of the colon and rectum often characterized by debilitating symptoms such as stomach pain, diarrhea, intestinal bleeding and weight loss. In addition to the morbidity, the risk of colitis-associated colon cancer (CAC) in UC remains higher than that of the general population. H_2S, a gasotransmitter, has been shown to exhibit anti-inflammatory effects in various disease states such as acute pancreatitis, sepsis, and asthma. In mammals, cystathionine-gamma-lyase (CSE) and cystathionine-beta-synthase (CBS) are the major enzymes implicated in H_2S production during transsulfuration. Others have shown that CSE is a major endogenous H_2S-producing enzyme in the normal colons of mice and rats, protecting the mucosa from inflammatory insults. However, little is known about the role CSE in human colitis and colitis-associated carcinogenesis. We have shown that CBS is elevated in human sporadic cancer and also is implicated in the progression of adenoma to adenocarcinoma in mouse models. We hypothesize that the protective effects of CSE are lost in human UC and the dysregulation of the transsulfuration enzymes are important in UC-associated carcinogenesis.

Methods: Fresh human tissue samples were obtained from patients undergoing colonoscopic screening for cancer in normal and UC in compliance with protocols approved by the UTMB Institutional Review Board. Immunostaining followed by confocal microscopy was used to analyze the CSE and CBS expression in situ. Fluorescence

was then quantified using the ImageJ software. Statistical analysis was performed using SPSS. To investigate CAC, we used the chronic model of inflammation combined with a mutagen. The colonic mucosal irritant, DSS, was administered for three, one-week cycles and combined with a single dose of the mutagen AOM to induce CAC. CSE null mice and wild type Sv129/B6 (WT) mice were treated over a course of 80 days with this protocol. Animals treated with DSS or AOM alone were used as controls. Real time RT-PCR, Western blot and confocal microscopy was used to assess the gene expression. Size, number, and time interval to tumor formation, as well inflammation were assessed.

Results: Using *in-situ* confocal microscopy of three normal and five UC patient's colons, we discovered that CSE protein expression was significantly lower in UC when compared with normal mucosa (Student's t-test, $p < 0.0001$). A decrease in CSE expression was also observed at the mRNA level. The inflammation characteristic of UC was validated by an increased presence of $CD45^+$ hematopoietic cells. Importantly, similar to human UC, a significant decrease in the colonic CSE expression was observed in WT animals treated either with DSS alone or AOM-DSS. Genetic abrogation of CSE expression using CSE null animals in the AOM-DSS model of accelerated CAC, resulting in the increase of time to tumor development and an increase in both tumor size ($p<0.001$) and number ($p<0.001$), compared to WT animals. Interestingly, we observed a dramatic increase in the basal level of cancer-promoting cytokine, IL-6, in the colon of untreated CSE null animals compared to WT. Additionally, concomitant with a decrease in CSE, there was an increase in CBS protein expression in all the mouse colon tumors compared to normal colon mucosa—consistent with our previous findings of human colon cancer. Finally, loss of CSE promotes tumor spread, as indicated by the presence of liver metastases in the CSE null mice, an exceedingly rare finding for the AOM-DSS mouse model of CAC.

Conclusion: We show that human UC is associated with a reduced level of CSE, and in a genetically modified mouse model show that loss of CSE accelerates colitis-associated carcinogenesis and metasta-

sis. Furthermore, mouse colon cancer is associated with upregulation of CBS. Taken together, our human and murine data suggest that dysregulation in the transsulfuration pathway may be critical contributor to CAC development and may serve as a potential target for diagnostic and/or therapeutic intervention.

References:

1. Langan R.C., Gotsch P.B., Krafczyk M.A., Skillinge D.D. Ulcerative colitis: diagnosis and treatment. Am Fam Physician. 2007;76(9):1323-30.

2. Popivanova B.K., Kitamura K., Wu Y. et al. Blocking TNF-alpha in mice reduces colorectal carcinogenesis associated with chronic colitis. J Clin Invest. 2008;118(2):560-70.

3. Kappelman M.D., Rifas-shiman S.L., Porter C.Q. et al. Direct health care costs of Crohn's disease and ulcerative colitis in US children and adults. Gastroenterology. 2008;135(6):1907-13.

4. Chen Y.H., Wu R., Geng B. et al. Endogenous hydrogen sulfide reduces airway inflammation and remodeling in a rat model of asthma. Cytokine. 2009;45(2):117-23.

5. Mattar M.C., Lough D., Pishvaian M.J., Charabaty A. Current management of inflammatory bowel disease and colorectal cancer. Gastrointest Cancer Res. 2011;4(2):53-61.

6. Hirata I., Naito Y., Takagi T. et al. Endogenous hydrogen sulfide is an anti-inflammatory molecule in dextran sodium sulfate-induced colitis in mice. Dig Dis Sci. 2011;56(5):1379-86.

7. Zhang G., Wang P., Yang G., Cao Q., Wang R. The inhibitory role of hydrogen sulfide in airway hyperresponsiveness and inflammation in a mouse model of asthma. Am J Pathol. 2013;182(4):1188-95.

8. Szabo C., Coletta C., Chao C. et al. Tumor-derived hydrogen sulfide, produced by cystathionine-β-synthase, stimulates bioenergetics, cell proliferation, and angiogenesis in colon cancer. Proc Natl Acad Sci USA. 2013;110(30):12474-9.

9. Motta J.P., Flannigan K.L., Agbor T.A. et al. Hydrogen sulfide protects from colitis and restores intestinal microbiota biofilm and mucus production. Inflamm Bowel Dis. 2015;21(5):1006-17.

Activation of Nrf2/Trx-1 pathway by hydrogen sulfide attenuates lipopolysaccharide-induced endoplasmic reticulum stress in rat hepatocytes

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This study is to investigate the role of transcription factor NF-E2-related factor 2 (Nrf 2) and its downstream gene thioredoxin-1 (Trx-1) in hydrogen sulfide(H_2S) protection against LPS-induced endoplasmic reticulum stress in rat hepatocytes. H_2S donor **s**odium hydrosulfide(NaHS) and lipopolysaccharide(LPS) led to Nrf2 protein expression upregulation in cytosol and nucleus respectively, ratio of Nrf2 protein in nucleus and cytosol increased in LPS exception for NaHS administration.Pretreatment of NaHS 1h prior to LPS administration induced significantly increased Nrf2 protein expression in nucleus. 80% of Nrf2 protein expression was abolished following Nrf2 siRNA transfection for 12h,accompanying remarkedly decreased HO-1 and Trx-1 protein expression of Nrf2 downstream genes. Nrf2 siRNA transfection in part reversed the NaHS protection from LPS-induced endoplasmic reticulum stress with enhanced GRP78,CHOP and caspase-12 protein expressions and decreased cell viability. In separate experiment, administration of LPS for 12h caused Trx-1 and TrxR protein expression upregulation in contrast TXNIP protein expression downregulation as well as increased GRP78,CHOP and caspase-12 protein expressions and decreased cell viability.Trx-1 siRNA transfection aggravated LPS-induced the enhanced GRP78,CHOP and caspase-12 protein expressions and further decreased cell viability. 50~200μmol/L NaHS significantly enhanced LPS-induced Trx-1 and TrxR protein expression upregulation but suppressed the increased GRP78,CHOP and caspase-12 protein expressions and the decreased cell viability. Aforementioned effects of NaHS were in part reversed by Trx-1 siRNA transfection.These data implied that exogenous hydrogen sulfide attenuated LPS-induced endoplasmic reticulum stress in rat hepatocytes via activation of

Nrf2/Trx-1 pathway.

Slow sulfide donor GYY4137 differentiates NG108-15 neuronal cells through different intracellular transporters than dbcAMP

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IP3 receptors (IP3Rs) are crucial calcium release channels localized mainly on the endoplasmic reticulum (ER) that release calcium from these calcium stores. Up to now, three types of these receptors were identified, cloned and characterized – type 1, 2 and 3. Although several functions were attributed to these receptors, their ability to induce apoptosis and a change of cell´s plasticity belongs to the most important functions in cancer cells. H2S is known to modulate a variety of cellular functions. Nevertheless, its physiological consequences due to IP3Rs modulation are still not completely clear. We have shown that IP3Rs were modulated by H2S, since a slow sulfide donor GYY4137 was able to upregulate expression of the IP3R1 and IP3R2 on both, mRNA and protein levels in HeLa and ovarian cancer cells A2780 cells and increased number of apoptotic cells. Moreover, H2S-induced cytosolic calcium was elevated and reticular calcium was depleted in a concentration-dependent manner, partially through IP3Rs. Interestingly enough, in hypoxic conditions that significantly worsen tumor prognosis, H2S-induced increase in IP3Rs and apoptosis was even more pronounced. On the other hand, in glioblastoma NG-108 cells, H2S released from the GYY4137 was able to significantluy change plasticity, similarly to the cAMP boost. Due to the GYY4137 treatment, these cells were change to the neuronal fenotype, which was accompanied by a rapid decrease in sarco/endoplasmic ATPase. Thus, H2S play an important role in cancer cell´s modulation, where H2S-IP3Rs interaction is involved.
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H2S Augmentation Rescues Hypobaric Hypoxia Induced Perturbation of Functional Hyperemia

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Hypobaric Hypoxia (HH) occurs at high altitude and is associated with multiple pathophysiological conditions like Acute Mountain Sickness (AMS), cognitive deficit etc (Wilson et al., 2009). Recently, role of H2S in hypoxic cerebral autoregulation has been established (Morikawa et al., 2012), however, its effect in context to HH induced cerebral vascular homeostasis is not yet known. The present study was undertaken to investigate whether H2S regulates HH associated conditions. We used zinc precipitation assay to measure H2S levels, interestingly, a significant lowering was observed after HH exposure. Further, to understand how functional hyperemia, an important phenomenon associated with increase in neuronal activity with increase in local cerebral blood flow (CBF) is perturbed during HH. We utilized 'Whisker-Stimulation' protocol and recorded changes in CBF in barrel cortex area after rhythmic whisker stimulation. Intriguingly, a direct correlation between reduction in H2S levels and functional hyperemia (average CBF change after whisker stimulation represented as ΔBPU), concomitant with disturbed Glio-Vascular homeostasis in brain was recorded under chronic HH conditions. Following this, pre-treatment with NaHS (4 mg/kg/day, i.p.), an H2S donor, under HH condition was found to rescue functional hyperemia , which was associated with significant increase in NO and cGMP levels in the brain. Thus, indicating role of H2S augmentation in controlling ‘hypoxic cerebral autoregulation’ through cGMP mediated pathway. Additionally, H2S treatment also restored HH induced perturbation of Glio-Vascular homeostasis. These results, taken together, suggest a critical role of H2S in regulation of functional hyperemia besides Glio-Vascular homeostasis likely by vasodilation of brain vasculature.

References:

1. Morikawa T., Kajimura M., Nakamura T., Hishiki T., Nakanishi T., Yukutake Y., Nagahata Y., Ishikawa M., Hattori K., Takenouchi T., Takahashi T., Ishii I., Matsubara K., Kabe Y., Uchiyama S., Nagata E., Gadalla M.M., Snyder S.H., Suematsu M. Hypoxic regulation of the cerebral microcirculation is mediated by a carbon monoxide-sensitive hydrogen sulfide pathway. Proceedings of the National Academy of Sciences of the United States of America. 2012;109:1293-1298.

2. Wilson M.H., Newman S., Imray C.H. The cerebral effects of ascent to high altitudes. Lancet Neurol. 2009;8:175-191.

Deficiency in renal erythropoietin production is a result of impaired endogenous hydrogen sulfide

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Introduction: Anemia affects 90% of hemodialysis patients and is a tremendous concern for patients and healthcare providers (1). Despite the persistence of renal hypoxia in chronic kidney disease (CKD), interstitial fibrosis associated with CKD leads to a dysfunction in erythropoietin (EPO) production in the kidney. As a result, anemic CKD patients are often treated with erythropoietin stimulating agents (ESAs), which are associated with many adverse effects including hypertension and drug resistance, implicating a need for alternative treatments (2,3). Interestingly, hydrogen sulfide (H2S), an endogenously produced renal oxygen sensor molecule, is found at lower levels in anemic patients (4). Taken together, we postulate that H2S may be the primary mediator of EPO production during hypoxia. Our hypothesis was tested using both in vivo and clinical patient samples.

Results: After 72 hours of 11% hypoxia, C57BL/6 CSE-/- mice (mice deficient in cystathionine γ lyase, an enzyme responsible for

endogenous H2S production), demonstrated significantly lower levels of hemoglobin, EPO, and other HIF regulated genes when compared to wildtype C57BL/6 mice; this was rescued upon twice daily injection of 4mM/kg sodium sulfide (Na2S). This phenomenon was also reflected in the clinical setting, where urinary H2S and thiosulfate levels of anemic CKD patients were significantly lower than CKD patients who were not anemic. As urinary nitrate and nitrite levels (measured by HPLC) did not differ between the two groups, this suggests that H2S, rather than nitric oxide, is responsible for EPO deficiency.

Conclusion: Our findings demonstrate, for the first time, the interplay between the production and actions of H2S during hypoxia and EPO production. This suggests that H2S donor molecules may potentially be a novel therapeutic strategy in treating anemic CKD patients

References:

1. Eckardt K.U. Anaemia in end-stage renal disease: pathophysiological considerations. Nephrol Dial Transplant. 2011;16:2-8.

2. Change Y.T., San S.Y., Lin S.L. Seeking for a way to revive erythropoietin production in chronic kidney disease. J Formos Med Assoc. 2013;12:657-658

3. Hung S.C., Lin Y.P., Tarng D.C. Erythropoiesis-stimulating agents in chronic kidney disease: what have we learned in 25 years? J Formos Med Assoc. 2014;113:3-10.

4. Perna A.F., Luciano M.G., Ingrosso D., Raiola I., Pulzella P., Sepe I. et al. Hydrogen sulfide, the third gaseous signaling molecule with cardiovascular properties, is decreased in hemodialysis patients. 2010;20:S11-S14.

Hydrogen sulfide ameliorates renal graft necrosis associated with prolonged cold storage and improves survival after allogeneic renal transplantation

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Organ procurement is inherently associated with ischemia-reperfusion injury (IRI), resulting from loss and subsequent restoration of blood flow, which is detrimental to short- and long-term graft function and survival (1-3). Treatment of donor organs with small molecules such as hydrogen sulfide (H2S) is a novel method of modulating prolonged IRI during renal transplantation (4). We postulated that H2S treatment during prolonged cold storage could mitigate IRI-induced renal allograft injury following allogeneic renal transplantation (RTx). Following bilateral native nephrectomy, recipient Lewis rats underwent RTx with kidneys obtained from Brown Norway donor rats that were flushed with either University of Wisconsin preservation solution (UW group; n=8) or UW + 150 μM NaHS (H2S group; n=8) and stored for 24 hours at 4°C in the same solution. Sham surgeries (midline incision only; n=5) were also performed and animals were monitored for 14 days to assess allograft function and survival. An additional subset of donor kidneys in each treatment group (n=6 per group) were perfused with 5 μM Ethidium Homodimer-1 (EthD-1) immediately following cold storage and analyzed via fluorescent microscopy for in situ characterization of tissue necrosis. ATP levels were also measured to assess the metabolic state of donor kidneys following cold storage. H2S treated animals exhibited significantly improved ($p<0.05$) survival and markedly decreased serum creatinine compared to UW treatment alone. Donor kidneys supplemented with H2S exhibited significantly decreased ($p<0.05$) EthD-1 staining and

markedly improved ATP levels compared to UW treated kidneys immediately following cold storage. H2S treatment mitigates IRI associated with prolonged cold storage and acutely improves subsequent allograft function and survival. H2S appears to limit tissue necrosis and may maintain cellular metabolism during prolonged cold storage.

H2S treatment could represent a novel and cost-effective method of protecting kidneys during transplantation and improving clinical transplant outcomes.

References:

1. Wilhelm M.J., Pratschke J., Laskowski I. et al: Ischemia and reperfusion injury. Transplant Rev 2003;17:140.

2. Salahudeen A.K., Haider N., May W. Cold ischemia and the reduced long-term survival of cadaveric renal allografts. Kidney Int. 2004;65:713.

3. Quiroga I., McShane P., Koo D.D. et al: Major effects of delayed graft function and cold ischaemia time on renal allograft survival. Nephrol Dial Transplant. 2006;21:1689.

4. Lobb I., Sonke E., Aboalsamh G. and Sener A. Hydrogen sulphide and the kidney: important roles in renal physiology and pathogenesis and treatment of kidney injury and disease. Nitric Oxide-Biol Ch. 2015;46:55

Hydrogen sulphide induces HIF-1α and Nrf2 in THP-1 macrophages

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The transcription factor HIF-1α (hypoxia inducible factor-1α) regulates the adaptive response of cells to hypoxia and oxidative stress. In addition, an important regulatory role for HIF-1α in immune reactions and inflammation is suggested. The present study attempts to investigate the effect of the gaseous signalling molecule hydrogen sulphide (H2S) on HIF-1α in THP-1 macrophages using the slow H2S releasing donor GYY4137. We found that H2S induced HIF-1α protein accumulation in THP-1 macrophages in a concentration-dependent manner. Western blot analysis of cell fractions showed that HIF-1α protein translocates into the nucleus and leads to an increase of its target protein glucose transporter-1 (GLUT-1). Activation of nuclear factor-αB (NF-αB), as well as secretion of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), were reduced in the presence of H2S. These findings indicate that HIF-1α accumulation due to H2S was not triggered by the NF-αB pathway. The antioxidant pathway Nrf2/HO-1 (nuclear factor erythroid 2-related factor 2/heme oxygenase-1) was activated by H2S. Inhibition of the p38 mitogen-activated protein kinase (MAPK) reversed H2S mediated effects, suggesting that the p38 MAPK pathway may be involved in H2S induced HIF-1α/Nrf2 signalling pathways.

Gastroprotective effect of hydrogen sulfide and carbon monoxide against aspirin-induced damage: involvement of heme oxygenase-1 and transcription factor Nrf-2

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Constitutive heme oxygenase (HO)-2 and inducible HO-1 are two catalytically active enzymes involved in heme degradation leading to production of endogenous carbon monoxide (CO). It has been shown that CO exerts anti-inflammatory and protective activity within gastrointestinal (GI) tract. We investigated if CO producing HO-1 is regulated by nuclear factor (erythroid-derived 2)-like 2 factor (Nrf2) in gastric mucosa with aspirin (ASA)-induced injury and whether increased content of CO released from its donor, tricarbonyldichlororuthenium (II) dimer (CORM-2) can affect this NSAID-induced gastric damage. Moreover, we focused on possible role of hydrogen sulfide (H2S) in regulation of endogenous CO content in gastric mucosa. Male Wistar rats were pretreated with 1) vehicle (saline), 2) CORM-2 (5 mg/kg i.g.), 3) NaHS (5 mg/kg i.g.), 4) zinc protoporphyrin (ZnPP, 5 mg/kg i.p.) as an inhibitor of HO-1 or 5) hemin (5 mg/kg i.g.), an inducer of HO-1. After 30 minutes, ASA at a dose of 125 mg/kg was administered i.g. to induce gastric damage. The area of ASA-induced gastric mucosal damage was evaluated macroscopically 1 hr later by planimetry. Gastric blood flow (GBF) was measured by laser Doppler flowmeter. CO content in gastric mucosa was determined by gas chromatography. H2S production via CSE/CBS pathway was determined by methylene blue method. Protein and mRNA expression for HO-1, HO-2, Nrf-2 and induced by inflammation cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS) and IL-1β was measured by Western Blot and/or

real-time PCR, respectively.
Administration of ASA (125 mg/kg i.g.) caused severe gastric damage and significantly decreased GBF and CO content in gastric mucosa. Protein and mRNA expression for HO-1 and Nrf-2 was increased, while HO-2 expression was downregulated. CORM-2 decreased ASA-induced gastric damage and increased GBF while ZnPP or hemin did not. CO donor significantly ($p<0.05$) enhanced mRNA expression for HO-1 and Nrf-2, increased CO content in gastric mucosa and downregulated iNOS, COX-2 and IL-1β mRNA expression and inhibited H2S production. NaHS increased GBF and exerted protection against ASA-induced gastric damage but failed to affect CO content in gastric mucosa. However, pre-treatment with NaHS decreased HO-1 mRNA expression. We conclude that CO and H2S are involved in protection against ASA-induced gastric damage but these gaseous mediators acts independently each other via regulation of HO-1/Nrf-2 pathway. Increased gastric mucosal content of CO, produced by HO-1 expression and activity and released from CORM-2 protects gastric mucosa against ASA-induced gastric damage via an enhancement in GBF and anti-inflammatory properties of this gaseous mediator. This study was supported by a grant from National Science Centre in Poland (UMO-2014/15/N/NZ4/04564).

References:

1. Magierowski M., Jasnos K., Kwiecień S., Brzozowski T. Role of hydrogen sulfide in the physiology of gastrointestinal tract and in the mechanism of gastroprotection. Postepy Hig Med Dosw (Online). 2013 Mar 6;67:150-6.

2. Jasnos K., Magierowski M., Kwiecień S., Brzozowski T. Carbon monoxide in human physiology-its role in the gastrointestinal tract. Postepy Hig Med Dosw (Online). 2014 Jan 30;68:101-9. doi: 10.5604/17322693.1087527

3. Magierowski M., Jasnos K., Kwiecien S., Drozdowicz D, Surmiak M., Strzalka M., Ptak-Belowska A., Wallace J.L., Brzozowski T. Endogenous prostaglandins and afferent sensory nerves in gastroprotective effect of hydrogen sulfide against stress-induced gastric lesions. PLoS One. 2015 Mar 16;10(3):e0118972. doi: 10.1371/journal.pone.0118972.

4. Qin S.Y., Du R.H., Liu X.F., Xu G.L., Cao W. Nrf2 is essential for the anti-inflammatory effect of carbon monoxide in LPS-induced inflammation. Inflamm Res. 2015;64:537–548. doi: 10.1007/s00011-015-0834-9

5. Magierowski M., Magierowska K., Kwiecien S, Brzozowski T. Gaseous mediators nitric oxide and hydrogen sulfide in the mechanism of gastrointestinal integrity, protection and ulcer healing. Molecules. 2015 May 19;20(5):9099-123. doi: 10.3390/molecules20059099.

6. Magierowska K., Magierowski M., Hubalewska-Mazgaj M., Adamski J., Surmiak M., Sliwowski Z., Kwiecien S., Brzozowski T. Carbon Monoxide (CO) Released from Tricarbonyldichlororuthenium (II) Dimer (CORM-2) in Gastroprotection against Experimental Ethanol-Induced Gastric Damage. PLoS One. 2015 Oct 13;10(10):e0140493. doi: 10.1371/journal.pone.0140493.

7. Magierowska K., Magierowski M., Surmiak M., Adamski J., Mazur-Bialy A.I., Pajdo R., Sliwowski Z., Kwiecien S., Brzozowski T. The Protective Role of Carbon Monoxide (CO) Produced by Heme Oxygenases and Derived from the CO-Releasing Molecule CORM-2 in the Pathogenesis of Stress-Induced Gastric Lesions: Evidence for Non-Involvement of Nitric Oxide (NO). Int J Mol Sci. 2016 Mar 24;17(4).pii: E442. doi: 10.3390/ijms17040442.

Materials Approaches toward Targeted Delivery of Hydrogen Sulfide: Polymer Nanoparticles and Peptide-Based Hydrogels

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The majority of biological studies on hydrogen sulfide (H2S) have been carried out with systemically administered small molecule H2S donors, most of which have little tissue specificity, fast release, and the potential for off-target effects. We address these shortcomings by developing new materials with the potential for a high degree of control over the rate and location of H2S delivery. Based on the S-aroylthiooxime functional group, [Foster et al., 2014] which releases H2S in response to a thiol trigger, we have developed two new materials-based approaches for H2S delivery. The first approach, which is suitable for systemic delivery, includes nanoparticles based on amphiphilic polymers. [Foster and Matson, 2014] The amphiphilic polymers include a hydrophobic segment that drives self-assembly in water to form nanoparticles. The rate of H2S release from the nanoparticles can be controlled by modifying several parameters, including the polymer block lengths and the conditions of assembly. Our biological studies on this system focus on cancer treatment using H2S delivered at a controlled rate through delivery of nanoparticles. The second approach is suitable for localized delivery and is based on short peptides with appended S-aroylthiooximes that form robust hydrogels in water. [Carter, 2015] Gelation occurs immediately upon charge screening, making these gels suitable for in vivo delivery to release H2S specifically at an area of interest. The peptide gels disassemble upon H2S release over the course of several hours into degradable oligopeptides. We are measuring the pro-angiogenic response of endothelial cells in response to H2S released at various rates in hydrogels with varying moduli.

Effect of Treatment with AP39, a Mitochondria-targeted Slow-H2S-releasing Drug, on Mitochondrial Respiration in Resuscitated Murine Polytrauma

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Introduction: The aim of this project was to investigate if exogenous H2S supplementation, using the drug AP39, can maintain mitochondrial respiration in anaesthetized and mechanically ventilated mice undergoing trauma. Impairment of mitochondrial function by oxidative and nitrosative stress was found to be crucially involved in the development of posttraumatic multiple organ failure1,2. H2S in low doses, serving as an electron donor for the mitochondrial electron transfer system, can stimulate mitochondrial respiration3. The administration of H2S by the slowly-releasing drug AP39 might be advantageous, since the compound contains a mitochondria-targeted motif4. AP39 indeed proved to elevate mitochondrial H2S levels and to have protective effects on mitochondria in endothelial cells in vitro4. The effects of AP39 on mitochondrial respiration have not yet been investigated in an in vivo model. Methods: C57/BL6 mice underwent blast wave-induced blunt chest trauma and directly afterwards were surgically instrumented to allow for mechanical ventilation and monitoring of hemodynamic and metabolic parameters. Hemorrhage was induced by blood withdrawal to a target mean arterial pressure of 35 mmHg and maintained for 1 h. Resuscitation involved fluid administration,

noradrenaline infusion (n=10), and, for two of the groups, infusion of AP39 (100 nmol/kg (n=8) or 10 nmol/kg (n=3)), chosen based on previous studies in mice5. Four hours later animals were sacrificed and mitochondrial respiration of kidney, liver, heart and diaphragm was investigated via high-resolution respirometry.

Results: Oxygen consumption during oxidative phosphorylation at saturating ADP concentrations as well as in the leak state and the uncoupled state were analysed. AP39 treatment was found to have no effect on these parameters. The activity of Complex I, II and IV alone were unaffected as well. No differences in the mitochondrial ATP production-related oxygen consumption could be determined between the AP39 treated and untreated mice.

Conclusion: The mitochondrial respiration of kidney, liver, heart and diaphragm remained unaffected by high-dose and low-dose AP39 treatment in murine polytrauma. Due to its novelty so far only scarce data are available on the efficacious dosage of the compound, thus the chosen doses might not be optimal to achieve the potentially beneficial effect of AP39 in this model. 100 nmol/kg AP39 was not well tolerated by the animals and showed possible toxic effects. 10 nmol/kg AP39 was better tolerated by the animals, but the timeframe of the treatment might be too short to exert its effect on mitochondrial function.
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References:

1. Brealey D. et al. Mitochondrial dysfunction in a long-term rodent model of sepsis and organ failure. Am J Physiol Regul Integr Comp Physiol. 2004;286:491-497.

2. Brealey D. et al. Association between mitochondrial dysfunction and severity and outcome of septic shock. Lancet. 2002;36:219-23.

3. Szabo C. et al. Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part I. Biochemical and physiological mechanisms. Br J Pharmacol. 2014;171:2099-2122.

4. Szczesny B. et al. AP39, a novel mitochondria-targeted hydrogen sulfide donor, stimulates cellular bioenergetics, exerts cytoprotective effects and protects against the loss of mitochondrial DNA integrity in oxidatively stressed endothelial cells in vitro. Nitric Oxide. 2014;41:120-130.

5. Ikeda K. et al. Mitochondria-targeted hydrogen sulfide donor AP39 improves long-term outcomes after cardiac arrest in mice. Nitric Oxide 2015;49:90-96.

Porcine Coronary Artery CSE, CBS, and 3-MST Expression after Resuscitated Septic Shock with Pre-existent Atherosclerosis

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Introduction: A down-regulation of the H2S-catalyzing enzymes cysthathionine- γ -lyase (CSE) and cysthathione- β -synthase (CBS) is associated with chronic cardiovascular pathology, e.g. hypertension, and atherosclerosis. Nevertheless, equivocal data are available on both the expression and the function of these enzymes in coronary arteries (CA).3-7 Septic patients with CA disease (CAD) are reported to present with impaired myocardial compliance in comparison to patients without CAD.1 Thus to understand the endogenous source for H2S synthesis in the CA, we investigated pigs with(out) pre-existing cardiovascular co-morbidity in septic shock.

Methods: Anesthetized and instrumented FBM (familial LDL-receptor-/-) pigs with high fat diet-induced hypercholesterolemia and atherosclerosis2 underwent poly-microbial septic shock (n=5) induced by inoculation of autologous faeces into the abdominal cavity, or sham procedure (n=5), and subsequently received intensive care therapy for 24 hours. German domestic pigs (YGP, n=5) were used as native controls. CSE, CBS and 3-MST expression was quantified by immunohistochemistry (densitometric image analysis, Zeiss Axiovision) of formalin-fixed paraffin sections from CA.

Results: CSE expression was decreased in the media of the CA due to the CAD 11 (9;14) vs 7 (6;9) ·107 and even more pronounced with combined CAD and sepsis 2 (2;3) ·107 (p=0.003). CBS protein was

not detected in the media of any of the CA examined, but was seen localized to the adventitia. Surprisingly, moderate CBS expression was found only in the atheromatous plaques of the septic CA. 3-MST expression was not found in any of the CA.

Conclusion: Decreased CSE expression in porcine CA was found in the atherosclerotic co-morbidity group and this decrease was even more pronounced in septic group. Endogenously synthesized H2S not only protects cardiac and vascular tissues from ischemic challenges but is also a potent vasodilator.5,6 Diminished endogenous H2S release may be implicated in the lower cardiac output and decreased oxygen transport reported for septic patients with CAD.1

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References:

1. Raper R.F. et al. The effects of coronary artery disease on cardiac function in nonhypotensive sepsis. Chest. 1988;94:507-511.

2. Thim T. et al. Familial hypercholesterolaemic downsized pig with human-like coronary atherosclerosis: a model for preclinical studies. EuroIntervention. 2010;6:261–268.

3. Cheang W.S. et al. 4-Aminopyridine-sensitive K channels contributes to NaHS-induced membrane hyperopolarization and relaxation in the rat coronary artery. Vascul. Pharmacol. 2010;53:94-98.

4. Casalini E.D. et al. Contribution of hydrogen sulfide to the control of coronary blood flow. Microcirculation. 2014;21:104-111.

5. Hedegaard E.R. et al. KV7 channels are involoved in hypoxia-induced vasodilation of porcine coronary arteries. Br. J. Pharmacol. 2014;171:69-82.

6. Chai Q. et al. Hydrogen sulfide impairs shear stress-induced vasodilation in mouse coronary arteries. Pflugers Arch. Eur. J. Physiol. 2015;467:329-340.

7. Kuo M.M. et al. MPST but not CSE is the primary regulator of hydrogen sulfide production and function in the coronary artery. Am J Physiol Heart Circ Physiol. 2016;310:H71–H79.

Modulation of Rat Hemodynamic Parameters by Intravenous Administration of Hydrogen Sulfide

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The arterial pulse waveform (APW) gives useful information on the cardiovascular system. Details of APW reflect functions of all components of cardiovascular system, including heart function, vessel elasticity, intracellular signaling through different pathways, conductivity system, function of membrane channels, etc. Therefore, to elucidate complex biological properties of H2S, we studied its effect on 13 rat hemodynamic parameters, which were obtained from APW.

The right jugular vein of anesthetized Wistar rats was cannulated for Na2S (H2S-donor) administration. The left carotid artery was cannulated, and fiber-optic pressure transducer was used to detect APW at high resolution. Na2S (12 μmol/kg) had transient (0.5-5 min) effects on hemodynamic parameters. It decreased systolic and diastolic blood pressure; increased pulse pressure; decreased heart rate; increased dP/dtmax; decreased relative level of dP/dtmax; increased negative dP/dtmin; increased negative relative level of dP/dtmin; increased systolic and diastolic area; decreased dicrotic notch relative level; increased dicrotic notch delay and decreased dicrotic notch relative delay. The results may stimulate further studies to assign the H2S induced changes of the hemodynamic parameters to the function of specific component(s) of cardiovascular system and elucidate its molecular mechanism of action.

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β_3 adrenergic receptor activation relaxes human corpus cavernosum via cGMP/ hydrogen sulfide-dependent mechanism

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Introduction Human penile erection is a result of several complex neuronal and hemodynamic mechanisms. The balance between contracting and relaxant factors represent the key issue to achieve penile erection. It is now well accepted that nitric oxide (NO) plays a major role in induction and maintenance of erection resulting in sinusoids expansion and increased intracavernous pressure (Burnett 1995;Khan et al. 2000). However, alternative relaxing pathways have been widely described (Cirino et al. 2006; Khan et al. 2000; Jin and Burnett 2006). Indeed, it has been reported that β_3 adrenoceptor are expressed in human corpus cavernosum (HCC) and are localized mainly in smooth muscle cells (Cirino et al., 2003). β_3 adrenoceptor stimulation relaxes HCC strips in a cyclic guanosine monophosphate (cGMP)-dependent and NO-independent mechanism (Cirino et al. 2003). Therefore, β_3 adrenoceptors play a physiological role in penile erection, although the mechanism is still unclear. More recently, hydrogen sulfide (H_2S) has been suggested as a relaxant signal molecule involved in penile erection (d'Emmanuele di Villa Bianca et al., 2009). Cystathionine-β-synthase (CBS) and cystathionine- γ-lyase (CSE) are constitutively expressed in HCC but CSE rather than CBS is more abundant in human penile tissue (d'Emmanuele di Villa Bianca et al., 2009). Additionally, it has been demonstrated that H2S inhibits phosphodieserase increasing cGMP levels (Bucci et al., 2010). Therefore a link between cGMP and H2S exists.

Aim To investigate the possible involvement of H_2S pathway in HCC and penile artery relaxation induced by β_3 receptor stimulation.

Methods A relaxation response-curve to BRL37344, a β_3 selective agonist, has been performed in HCC strips or penile artery rings in presence of CSE inhibitor. H_2S generation and cGMP content has been measured in HCC and in human penile artery samples incubated with BRL37344. In order to better define the mechanism a pharmacological approach has been operated.

Results BRL37344 relaxes in a concentration dependent manner HCC strips as well as penile arterial rings. BRL37344-induced effect is significantly reduced by CSE inhibitor. The stimulation of β_3 adrenoceptor significantly increases H_2S production in both HCC strips or penile artery samples. This effect is significantly reduced by either protein kinase G or CSE inhibitor, and by β_3 antagonist. Finally the BRL-increase in cGMP is significantly reduced by CSE inhibition.

Conclusions BRL37344-induced relaxation in HCC and penile artery occur by cGMP/H_2S-dependent mechanisms. Mirabegron®, a β_3 agonist, has been recently approved by FDA for lower urinary tract symptoms (LUTS) treatment. LUTS and erectile dysfunction are strongly associated in men and share several possible pathogenetic mechanisms. Therefore, a therapy with β_3 agonists may open new frontiers in the treatment of patients affected by LUTS associated with erectile dysfunction.

References:

1. Burnett, A.L. Role of nitric oxide in the physiology of erection. Biol Reprod. 199;52(3):485-489.

2. Khan, M.A., Morgan R.J. and Mikhailidis D.P. The molecular basis of penile erection. Curr Med Res Opin. 2000;16 Suppl1:s21-30.

3. Cirino G., Sorrentino R., d'Emmanuele di Villa Bianca R., Popolo A., Palmieri A., Imbimbo C., Fusco F., Longo N., Tajana G., Ignarro L.J., Mirone V. Involvement of beta 3-adrenergic receptor activation via cyclic GMP- but not NO-dependent mechanisms in human corpus cavernosum function. Proc Natl Acad Sci USA. 2003;100(9):5531-6.

4. Cirino G., Fusco F., Imbimbo C. and Mirone V. Pharmacology of erectile dysfunction in man. Pharmacol Ther. 2006;111(2):400-423.

5. Jin L. and Burnett A.L. RhoA/Rho-kinase in erectile tissue: mechanisms of disease and therapeutic insights. Clin Sci. 2006;110(2):153-165.

6. Cai W.J., Wang M.J., Moore P.K., Jin H.M., Yao T. and Zhu Y.C. The novel proangiogenic effect of hydrogen sulfide is dependent on Akt phosphorylation. Cardiovasc Res. 2007;76(1):29-40.

7. D'Emmanuele di Villa Bianca R., Sorrentino R., Maffia P., Mirone V., Imbimbo C., Fusco F., De Palma R., Ignarro L.J., Cirino G. Hydrogen sulfide as a mediator of human corpus cavernosum smooth-muscle relaxation. Proc Natl Acad Sci USA. 2009106(11):4513-8.

8. Bucci M., Papapetropoulos A., Vellecco V., Zhou Z., Pyriochou A., Roussos C., Roviezzo F., Brancaleone V. and Cirino G. Hydrogen sulfide is an endogenous inhibitor of phosphodiesterase activity. Arterioscler Thromb Vasc Biol. 2010;30(10):1998-2004.

Cross-talk among H2S, NO, and CO in isolated rat pancreatic β-cells

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Aim: Nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H2S) are endogenously produced gasotransmitters, playing crucial roles in various cellular events. In pancreatic tissues, accumulated lines of evidence indicate that these gases are involved in pathophysiology of diseases including inflammation and diabetes. Regarding underlying mechanisms, while each bioactive gas exerts its function via respective cascade, recent studies implicate that these gases interplay in complicated ways, leading to diverse cellular effects by still unknown synergistic mechanisms. In the present study, an attempt was made to elucidate the cross-talk among the these gases, especially from a view point of potential effects on intracellular Ca2+, one of essential second messengers, that regulates various cellular functions in freshly isolated pancreatic islets and single b-cells of the rat.

Methods: Pancreatic islets were isolated enzymatically, purified, and dispersed into single b-cells. Immunofluorescent detection of gas-producing synthases was carried out in single b-cells with specific antibodies. Changes in intracellular Ca2+ concentration ([Ca2+]i) were examined fluorometrically using fluo-4. Cellular NO production was monitored using DAF-2. CO release to the incubation media of b-cells was estimated by carbonmonoxy myoglobin formation with CO-releasing molecule-2 (CORM-2) as a standard. Cellular H2S accumulation was examined using a polysulfide probe, SSP4. Basal insulin secretion from the intact islets was measured with a rat insulin ELISA kit.

Results: All major constitutive synthases for NO, CO, and H2S were

found to be expressed in single b-cells (Fig. 1A-D). CORM-2 and NaHS increased the NO production (Fig. 2A-D). CORM-2 and SNP elevated SSP4 fluorescence (Fig. 3A-D). The CO- and NO-induced H2S production was partially inhibited by hypotaurine, an H2S scavenger (Fig. 3E, F), suggesting that the fluorescence increase in SSP4 is associated with the H2S production. SNP and NaHS accelerated the CO production (Fig. 4A-C). In the absence of extracellular Ca2+, a calmodulin antagonist (SKF-7171A) significantly attenuated the NO production by CO or H2S (Fig. 5A, B) and the H2S increase by CO or NO (Fig. 5C, D), but the CO production by H2S or NO was unaffected (Fig. 5E, F). [Ca2+]i increase induced by H2S was significantly inhibited in the absence of extracellular Ca2+ but that by NO (except for by the highest concentration) or CO remained unchanged (Fig. 6A-D). NO dose-dependently stimulated the basal insulin release but CO dose-dependently inhibited it. H2S showed an insignificant effect on the basal insulin secretion from freshly isolated pancreatic islets (Fig. 6A-C).

Conclusions: It was presumed that one of three gases can produce other two gases. CO and NO were thought to induce the [Ca2+]i increase mainly via Ca2+ release from internal stores. In contrast, H2S was assumed to induce the [Ca2+]i increase, to a large extent, via the influx of extracellular Ca2+. Based upon these findings, we concluded that there is a synergistic cross-talk among three gases with respect to their production and intracellular Ca2+ dynamics, which result in diverse effects on the basal insulin secretion. The pivotal cross-talk among NO, CO and H2S most likely regulate cellular functions via the modulation of intracellular Ca2+ homeostasis in pancreatic b-cells of the rat.

References:

1. Moustafa A., Habara Y. Hydrogen sulfide regulates Ca2+ homeostasis mediated by concomitantly produced nitric oxide via a novel synergistic pathway in exocrine pancreas. Antioxid Redox Signal. 2014;20:747-758.

2. Moustafa A., Habara Y. A novel role for carbon monoxide as a potent regulator of intracellular Ca2+ and nitric oxide in rat pancreatic acinar cells. Am J Physiol Cell Physiol, 2014;307:C1039-C1049.

3. Moustafa A., Habara Y. Reciprocal interaction among gas-otransmitters in isolated pancreatic β-cells. Free Radic Biol Med. 2016;90:47-58.

Sodium thiosulfate prevents chondrocyte mineralization and reduces the severity of murine osteoarthritis

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Objectives: Calcium-containing crystals, which encompass hydroxyapatite (HA) crystals, participate in the pathogenesis of osteoarthritis (OA). Sodium thiosulfate (STS) has been shown to be an effective treatment in calcification disorders such as calciphylaxis and vascular calcification. This study investigated the effects and mechanisms of action of STS in chondrocyte calcification and in a murine model of OA.

Methods: Murine chondrocytes were treated with STS or with H2S donors (NaHS and GYY4137). Chondrocytes calcification and chondrocytes IL-6, MCP-1 and reactive oxygen species (ROS) production upon HA crystals stimulation were assayed. STS's effects on genes involved in calcification, inflammation and cartilage matrix degradation were studied by qRT-PCR. Finally, STS was administered in the menisectomy model of murine OA, and the effect on periarticular calcific deposits and cartilage degradation was investigated by micro-CTscan and histology.

Results: In vitro, STS prevented in a dose-dependent manner chondrocyte calcification as well as HA crystal-induced IL-6 and MCP-1 production by chondrocytes. STS also had an antioxidant effect by diminishing HA-induced ROS generation and abrogating HA-induced catabolic responses in chondrocytes. Most importantly, the anti-mineralizing and anti-inflammatory effects of STS can be reproduced with NaHS and GYY4137. In vivo, administration of STS ameliorated the histological severity of OA, by limiting the size of

new periarticular calcific deposits and reducing the severity of cartilage damage.

Conclusions: STS reduces the severity of periarticular calcification and cartilage damage in an animal model of OA via its inhibitory effects on chondrocyte calcification, and its attenuation of crystal-induced inflammation as well as catabolic enzymes and ROS generation. As STS anti-mineralizing and anti-inflammatory effects can be reproduced with NaHS and GYY4137, our study suggests that H2S donors may be of therapeutic interest as disease-modifying drugs in crystal-associated OA.

Chronic H2S treatment protects vascular function by reducing oxidative stress in diabetic mice

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Hydrogen sulfide is endogenously produced in vascular tissue, has vasoprotective properties and may be a useful therapeutic agent under conditions of increased oxidative stress. This study investigates whether chronic treatment with H2S via the fast donor NaHS could elicit a vasoprotective effect in diabetes, where there is known to be increased oxidative stress and endothelial dysfunction. Diabetes was induced in male C57 mice with streptozotocin (60mg/kg daily, ip for 10 days) and confirmed by elevated blood glucose and HbA1C levels. Following a further 2 weeks, mice were then treated with NaHS (100µmol/kg/day) for 4 weeks, then tissues collected. Acetylcholine mediated, endothelium dependent vasorelaxation, vasorelaxation to the NO donor sodium nitroprusside, NO bioavailability and eNOS expression were all significantly inhibited in diabetic aortae ($p<0.05$), but NaHS treatment restored these responses. Diabetes induced increased vascular superoxide generation via NADPH oxidase ($p<0.05$) and increased Nox2 expression ($p<0.05$), both these were inhibited by NaHS treatment. H2S bioavailability was decreased whilst cystathionine-γ-lyase (CSE) expression was increased in diabetes ($p<0.05$) but NaHS treatment restored these responses. These data show that chronic NaHS treatment protects endothelial function and both endogenous NO bioavailability and exogenous NO efficacy in this model of diabetes and vascular disease. NaHS treatment reduces oxidative stress in this model of diabetes by inhibiting vascular superoxide production via Nox2. Finally, H2S bioavailability is reduced in this model of diabetes, despite increases in CSE expression. Interestingly, both these effects are also ameliorated by NaHS treatment. Thus H2S treatment is beneficial in reversing endothelial dysfunction and oxidative stress in this model of diabetes.

A Novel Hydrogen Sulfide Releasing Compound as Potential Mimetic of Dietary Restriction in Caenorhabditis elegans

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Ageing is associated with the progressive accumulation of detrimental changes and increasing susceptibility to age-dependent diseases and death. There is emerging evidence that hydrogen sulfide (H2S) has profound effects on a wide range of physiological processes, including ageing [1] and ageassociated disorders [2, 4, 5, 7, 9]. GYY4137, a slow H2S release donor drug has been shown previously to promote longevity extension and delay deterioration of age-related physiological function in Caenorhabditis elegans (C. elegans) [8]. We have developed a novel H2S donor drug, FW1256 and examined for its effect on lifespan in C. elegans. FW1256 has been designed to have a higher H2S release rate than GYY4137. Our preliminary data showed that 500μM of FW1256 robustly prolonged lifespan of wild type C.elegans by 14% (26.2 ± 0.3 days, $P<0.0001$) compared to control (23.4 ± 0.3 days) in terms of mean lifespan and also promoted health span of wild type C.elegans significantly. Recently, Hine and colleagues have reported that induction of H2S synthesis is a necessary event for the lifespan extension effect of dietary restriction (DR) [10]. DR not only extends lifespan, but also prevents the progression of several age-associated diseases in many species [3, 6]. Hence, discovery of DR mimetic drugs that could replicate DR associated effects in living organisms is of great interest. Our preliminary data shows that the rate of development of wild type C.elegans exposed to 500uM of FW1256

is slower than untreated wild type C.elegans and is similar to eat-2 mutant C.elegans, a genetic model of DR. We have further investigated the possibility that FW1256 may act as a DR mimetic and replicate some of the beneficialeffects of DR in C. elegans.

References:

1. Miller D.L., Roth M.B. Hydrogen sulphide increases thermotolerance and life span in Caenorhabditis elegans. Proceedings of the National Academy Sciences of the United State of America. 2007;104:20618-20622.

2. Li L., Tellez M.S., Tan C.H., Whiteman M., Moore P.K. GYY4137, a novel hydrogen sulfide-releasing molecule, protects against endotoxic shock in the rat. Free Radical Biology & Medicine. 2009;47:103-113.

3. Fontana L., Partridge L., Longo V.D. Extending healthy lifespan—from yeast to humans. Science. 2010;328:321–326.

4. Hu L., Lu M., Tiong C.X., Dawe G.S., Hu G., Bian J. Neuroprotective effects of hydrogen sulfide on Parkinson's disease rat models. Aging cell. 2010;9(2):135-146.

5. Lee Z.W., Zhou J., Chen C.S., Zhao Y., Tan C.H., Li L., Moore P.K., Deng L.W. The slow-releasing Hydrogen sulfide donor, GYY4137, exhibits novel anti-cancer effects in vitro and in vivo. PLoS ONE. 2011;6(6):e21077.

6. Colman R.J., Beasley M.T., Kemnitz J.W., Johnson S.C., Weindruch R., Anderson R.M. Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys. Nature communications. 2013;5(3557).

7. Giuliani D., Ottani A., Zaffe D., Galantucci M., Strinati F., Lodi R., Guarini S. Hydrogen sulfide slows down progression of experimental Alzheimer's disease by targeting multiple pathophysiological mechanisms. Neurobiology of Learning and Memory. 2013;104:82-91.

8. Qabazard B., Li L., Gruber J., Peh M.T., Ng L.F., Srinivasan D.K., Rose P., Tan C.H., Dymock B.W., Wei F., Swain S., Halliwell B., Sturzenbaum S.R., Moore P.K. Hydrogen Sulfide is an endogenous regulator of aging in Caenorhabditis elegans. Antioxidants and redox signaling. 2013;20(16):2621-2630.

9. Polhemus D.J., Calvert J.W., Butler J., Lefer D.J. The Cardioprotective Actions of Hydrogen Sulfide in Acute Myocardial Infarction and Heart Failure. Scientifica. 2014;2014:768607.

10. Hine C., Harputlugil E., Zhang Y., Ruckenstuhl C., Lee B.C., Brace L. et al. Endogenous Hydrogen Sulfide Production Is Essential for Dietary Restriction Benefits. Cell. 2015;160:132–144.

Left Ventricular Function During Porcine Resuscitated Septic Shock With Pre-Existing Atherosclerosis

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Introduction: In sepsis, cardiac function is frequently depressed, termed septic cardiomyopathy, and characterised by decreased ejection fraction (EF) and compensatory ventricular dilatation to maintain stroke volume1. This adaptive cardiac response has mainly been demonstrated in patients or animals without pre-existing cardiac disease and/or without vasopressor support and is thought to be dependent on nitric oxide (NO)2. Atherosclerosis is a frequently encountered cardiac comorbidity on ICU and associated with reduced availability of NO5 and hydrogen sulphide (H2S)4. Therefore, we tested the hypothesis that pre-existing atherosclerosis alters the typical picture of sepsis-induced cardiomyopathy.

Methods: Faecal peritonitis was induced by inoculation of autologous faeces into the abdominal cavity in n=8 anesthetized and instrumented familial LDL-cholesterol-receptor-/- FBM pigs with high fat diet-induced hypercholesterolemia and atherosclerosis3 while n=5 animals underwent sham procedure. Pigs received intensive care therapy for 24 hours comprising crystalloids and norepinephrine infusion to maintain mean arterial pressure (MAP) at pre-sepsis values. Before, 12 and 24 hours after induction of peritonitis, we assessed LV function, systemic and pulmonary haemodynamics (LV pressure-conductance and pulmonary artery catheterisation). Data are medians (range).

Results: EF and left ventricular end diastolic volume (LVEDV) did not significantly change during sepsis. Dp/dt max significantly increased from 1571 (1341; 2025) mmHg/s to 3150 (1794; 5122)

mmHg/s after 24 hours of sepsis and the isovolumic relaxation constant τ significantly decreased from 28 (25; 38) ms to 18 (14; 23) ms. Cardiac nitrotyrosine formation significantly increased in septic animals compared to sham ($p<0.002$) whereas expression of cystathionine γ-lyase (CSE), an endogenous H2S-producing enzyme significantly declined ($p=0.01$).

Conclusion: The data of the present study are in conflict with previously published data from healthy animal models, most likely as a result of on-going resuscitation including norepinephrine infusion and intrinsic pathophysiologic processes of the pre-existing atherosclerosis. Moreover, increased nitrotyrosine formation and decreased expression of CSE suggests the implication of reduced bioavailability of NO and diminished endogenous H2S release in the pathophysiology of septic cardiomyopathy.

References:

1. Parker M. M. et al. Profound but reversible myocardial depression in patients with septic shock. Ann. Intern. Med. 1984;100:483–490.

2. Barth E. et al. Role of inducible nitric oxide synthase in the reduced responsiveness of the myocardium to catecholamines in a hyperdynamic, murine model of septic shock*. Crit Care Med. 2006;34:307–313.

3. Thim T. et al. Familial hypercholesterolaemic downsized pig with human-like coronary atherosclerosis: a model for preclinical studies. EuroIntervention. 2010;6:261–268 ().

4. Mani S. et al. Decreased endogenous production of hydrogen sulfide accelerates atherosclerosis. Circulation. 2013:127,2523–2534.

5. Li H., Horke S. & Förstermann U. Vascular oxidative stress, nitric oxide and atherosclerosis. Atherosclerosis. 2014;237:208–219.

Effects Of The Hydrogen Sulphide Donor AP39 During Resuscitated Murine Haemorrhagic Shock And Blunt Chest Trauma

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Introduction: Both the administration of exogenous H2S2,3 as well as the inhibition of H2S-producing enzymes1 have been reported to exert beneficial effects in haemorrhage. After cardiac arrest, the novel mitochondria-targeted H2S-donor AP39 improved survival and neurological outcome5. In the current study, we assessed the effects of AP39 during murine resuscitated haemorrhagic shock with blunt chest trauma.
Methods: Anaesthetised, spontaneously breathing mice (C57BL/6J) were subjected to blast wave-induced blunt chest trauma. After initiation of mechanical ventilation and instrumentation, mice underwent 1h of haemorrhage (mean arterial pressure (MAP)=35mmHg) followed by 4h of resuscitation comprising re-transfusion of shed blood, fluid administration, norepinephrine infusion to maintain MAP>50mmHg and lung-protective mechanical ventilation. Additionally, mice received a bolus injection of 100nmol/kg of the H2S donor AP39 (n=7) or vehicle (n=8) at the end of 1h of haemorrhage. Haemodynamics, lung mechanics, gas exchange and acid-base balance were measured together with visceral organ function. Data are medians (range).

Results: Administration of AP39 significantly decreased survival time (p=0.003). MAP was significantly lower in AP39-treated mice (45 (38;53) vs 58 (54;63) mmHg, p=0.007) despite significantly higher norepinephrine requirements (0.31 (0.25;0.63) vs 0 (0;0) µg x kg-1 x min-1, p=0.003). Metabolic acidosis was more pronounced with lower pH (7.21 (7.11;7.27) vs 7.34 (7.29;7.39), p=0.05) and more negative base excess (-10.1 (-16.8;-7.5) vs -5.5 (-10.5;-4.1) mmol/l, p=0.04) with AP39. AP39 treatment resulted in significantly decreased Horowitz index (375 (338;393) vs 450 (402;469) mmHg, p=0.01).

Conclusion: In contrast to the beneficial properties of AP39 following cardiac arrest5, AP39 exerted deleterious effects in our resuscitated model of haemorrhagic shock with blunt chest trauma, possibly due to its dose and time of administration. AP39-induced reduction of blood pressure4 may be responsible for the increased norepinephrine requirements and might at least partially explain the detrimental effects during traumatic haemorrhagic shock.
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References:

1. Mok Y.Y.P., Moore P.K. Hydrogen sulphide is pro inflammatory in haemorrhagic shock. Inflamm. Res. 2008:57:512–518.

2. Chai W. et al. Exogenous hydrogen sulfide protects against traumatic hemorrhagic shock via attenuation of oxidative stress. J. Surg. Res. 2012;176:210–219.

3. Issa K. et al. Compared effects of inhibition and exogenous administration of hydrogen sulphide in ischaemia-reperfusion injury. Crit Care. 2013;17:R129.

4. Tomasova L. et al. Effects of AP39, a novel triphenylphosphonium derivatised anethole dithiolethione hydrogen sulfide donor, on rat haemodynamic parameters and

chloride and calcium Cav3 and RyR2 channels. Nitric Oxide. 2015;46:131–144.

5. Ikeda K. et al. Mitochondria-targeted hydrogen sulfide donor AP39 improves neurological outcomes after cardiac arrest in mice. Nitric Oxide. 2015;49:90–96.

Metabolic Effects Of The Slow-Releasing Hydrogen Sulfide Donor GYY4137 During Resuscitated Porcine Septic Shock

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Introduction: We previously demonstrated that inhaled hydrogen sulfide (H2S) increased glucose oxidation in anaesthetized mice during hypothermia3 whereas normothermia or sepsis blunted this effect6. In contrast to the well-established metabolic effects of H2S in small mammals, the influence of H2S on metabolism in large animals is controversial. Both the absence of metabolic effects1 as well as reduction of energy expenditure2 was reported. Moreover, reduced H2S production is associated with development of atherosclerosis5. Therefore, we evaluated the metabolic effects of the slow-releasing H2S donor GYY4137 during resuscitated septic shock in swine with pre-existing atherosclerosis.

Methods: 12 and 18 hours after induction of fecal peritonitis, anaesthetized and instrumented LDL-cholesterol-receptor-/- pigs with high fat diet-induced hypercholesterolemia and atherosclerosis4 received either 10 mg·kg-1 of GYY4137 (n=9) or vehicle (saline, n=8). Animals underwent intensive care therapy for 24 hours comprising crystalloids, norepinephrine infusion to maintain mean arterial pressure (MAP) at pre-sepsis values and temperature control. Before, 12 and 24 hours after induction of peritonitis, we assessed calorimetric oxygen uptake, carbon dioxide production and blood gases. During infusion of stable, non-radioactively labelled

1,2,3,4,5,6-13C6-glucose, measurement of tracer enrichment in plasma by combined gas chromatography/mass spectrometry and analysis of 13CO2 enrichment in the expiratory gas by non-dispersive infrared spectrometry allowed quantification of endogenous glucose production and aerobic glucose oxidation. Data are expressed as medians (range).

Results: MAP, heart rate, noradrenaline requirements and body temperature were similar between GYY and vehicle. Glucose oxidation significantly increased in both groups after 12 and 24 hours compared to baseline values. However, GYY4137-treated pigs showed significantly higher tracer oxidation (44 (36; 51) vs 29 (23; 33) %, p=0.008) and total glucose oxidation rate (1.2 (0.9; 1.4) vs 0.5 (0.4; 0.6) mg·kg-1·min-1, p=0.005) after 24 hours, resulting in significantly higher requirement of exogenous glucose administration to maintain normoglycemia. While total CO2 production remained stable in 24 hours, CO2 production from glucose oxidation increased in both groups and was significantly higher in GYY-treated animals after 24 hours (27 (23; 38) vs 21 (19; 22) %, p=0.01). Arterial pH and base excess significantly decreased compared to baseline in the GYY group and pH was significantly lower compared to vehicle after 24 hours ($p<0.001$), whereas lactate levels were comparable.

Conclusion: In our study of resuscitated septic shock in swine with pre-existing atherosclerosis, GYY4137 shifted metabolism to preferential carbohydrate utilization under controlled normothermia. As a result of increased glucose oxidation, acidosis might be due to accumulating ketone bodies.

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References:

1. Haouzi P. et al. Respir Physiol Neurobiol. 2008;160:109.

2. Simon F. et al. Shock. 2008;30:359.

3. Baumgart K. et al. Crit Care Med. 2010;38:588.

4. Thim T. et al. EuroIntervention. 2010;6:261.

5. Mani S. et al. Circulation. 2013;127:2523.

6. Asfar P. et al. Critical Care. 2014;18:215.

Involvement of Prostaglandins and Acetylcholine in the Contractile Actions of Hydrogen Sulfide-Releasing Compounds in Bovine Isolated Irides

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We have evidence that hydrogen sulfide (H2S)-releasing compounds can relax pre-contracted porcine isolated irides, an effect that was dependent upon endogenous production of both H2S and prostaglandins, and on the activity of KATP channels (Monjok et al. Exp. Eye Res. 87: 612, 2008). However, H2S-releasing compounds had no contractile action on basal tone in porcine isolated irides. Purpose. The aim of the present study was to investigate the pharmacological actions of H2S-releasing compounds (NaHS and L-cysteine) on basal tone in bovine isolated irides. Furthermore, we studied role of prostaglandins and acetylcholine in the responses elicited by the H2S-releasing compounds on this tissue. Methods. Isolated bovine iris muscle strips were set up in an organ baths containing oxygenated Krebs buffer solution maintained at 37°C and gassed with 95% O2: 5% CO2. The muscle strips were set to a resting tension of 0.3 g and longitudinal isometric tension was recorded via a Grass FT03 force-displacement transducer and analyzed using PolyView Computer Software. Contractile responses were elicited by the H2S releasing compounds in the absence and presence cyclooxygenase (COX) inhibitors (flurbiprofen and indomethacin) or the muscarinic receptor blocker, atropine. Isolated bovine irides were also exposed to L-cysteine and tissues were prepared for PGE2 Enzyme-immunoassay using a well-established methodology. Results. NaHS (1 nM – 10 μM) and L-cysteine (100 nM - 1 mM) caused a concentration-dependent contraction of isolated bovine irides yielding EC50 values of 10 nM and 30 μM, respectively. Both COX inhibitors,

flurbiprofen (10 μM) and indomethacin (10 μM) abolished the contractile actions of NaHS and L-cysteine on this tissue. Likewise, atropine (1 nM – 10 nM) significantly ($p < 0.001$) antagonized the contractile response to NaHS and L-cysteine. L-cysteine (1 nM – 10 μM) elicited a concentration-dependent increase in PGE2 concentrations in both irides and in the incubation media indicating a washout of prostaglandins from tissues exposed to L-cysteine. The positive control, norepinephrine (1 μM) also caused an increase in tissue PGE2 concentrations over basal levels. Conclusions: We conclude that H2S-releasing compounds can elicit contraction of isolated bovine irides, an effect that is dependent upon the production of endogenous prostaglandins and the release of acetylcholine whose pharmacological actions can be blocked by the muscarinic receptor antagonist, atropine. Furthermore, differences exist between porcine and bovine species in the ability of their irides to elicit contractions to H2S-releasing compounds.

The reaction mechanism of nitroprusside with HS– (Gmelin process). Evidence of bound NOSH, NSOH, NOS–, NOS2–, NO•, HNO and N2O22– intermediates.

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Nitroprusside [Fe(CN)5(NO)]2− is an electrophilic anion reactive toward diverse types of O-, N-, and S-binding nucleophiles. HS− forms reversibly [Fe(CN)5(NOSH)]3−, an adduct that equilibrates very rapidly with its isomer [Fe(CN)5(NSOH)]3− and with the deprotonated [Fe(CN)5(NOS)]4− ion containing the nitrososulfide (thionitrite) ligand NOS− (Ired: λmax, 535 nm; ε = 6 ± 0.3 × 103 M−1 cm−1).[1,2,3] The stretching frequency νNO at 1370 cm−1 indicates a strongly reduced nitrosyl-species with formally a nitroxyl (NO−) ligand in the delocalized [S−N=O]− moiety. Ired reacts spontaneously by homolysis of the N-S bond giving sulfur radicals S·− which add to HS− forming highly reactive HS2.2− radicals favoring a catalytic generation of [Fe(CN)5(NO·)]3− and hydrodisulfide, HS2−. Ired adds sulfur through a HS2−/HS− interchange (transnitrosation reaction), giving [Fe(CN)5(NOS2)]4− (I550: λmax, 550 nm), also formed through the reaction of [Fe(CN)5(NO)]2− with HS2−. I550 contains the nitrosodisulfide (perthionitrite) ligand NOS2−, and transforms into the disulfur-bridged dimer, [(NC)5FeN(O)S-S(O)NFe(CN)5]6− (I575: λmax, 575 nm). Both I550 and I575 display an equilibrium redox network also comprising [Fe(CN)5(NO·)]3−/[Fe(CN)4(NO)·]2− and [Fe(CN)5(HNO)]3−, the one- and two-electron reduction products of [Fe(CN)5(NO)]2−, respectively. Under excess HS−, I575 forms a hyponitrite-bridged radical complex, [(NC)5FeN(OH)=(O·)NFe(CN)5]6− (I290: λmax, 290 nm) followed by disproportionation into the closed-shell bridged analog, with concomitant release of [Fe(CN)5(NO·)]3−. The hyponitrous/hyponitrite dimers can be subsequently reduced by HS− through proton-coupled electron transfers in the pH-range 7-12

leading to [Fe(CN)5(NH3)]3−, which forms [Fe(CN)5(H2O)]3−and free NH3. Early production of N2O arises at pH's > 11 through a competitive decomposition of the hyponitrite dimer, and in a slower way through NO-release from [Fe(CN)5(NO·)]3−, rapid dinitrosyl formation and disproportionation into [Fe(CN)5(NO)]2− and N2O. The overall reduction process involves a series of ligand-based redox reactions, from bound NO+ down to NH3. Free NO was detected in very low yields, and some HNO probably generates at the μM/nM level from the very inert [Fe(CN)5(HNO)]3− intermediate. S8 is the unique product of sulfide oxidation, and [Fe(CN)5(H2O)]3− decomposes slowly into [Fe(CN)6)]4− and free Fe(II) aqua-ions. The mechanism illustrates an active "NO/H2S" cross-talk involving three redox-states of nitrosyl (NO+, NO•, NO–/HNO) and sulfur (HS–, HS2–, So), and a discussion will be given on the biochemical significance of some of these species as potential signaling agents.[3]

References:

1. Quiroga S.L., Almaraz A.E., Amorebieta V.T., Perissinotti L.L., Olabe J.A. Addition and redox reactivity of hydrogen sulfides (H2S/HS–) with nitroprusside: new chemistry of nitrososulfide ligands. Chemistry. 2011 Apr 4;17(15):4145-56. PubMed PMID: 21404343.

2. Filipovic M.R., Eberhardt M., Prokopovic V., Mijuskovic A., Orescanin-Dusic Z., Reeh P., Ivanovic-Burmazovic I. Beyond H2S and NO interplay: hydrogen sulfide and nitroprusside react directly to give nitroxyl (HNO). A new pharmacological source of HNO. J Med Chem. 2013 Feb 28;56(4):1499-508. PubMed PMID:23418783.

3. Cortese-Krott M.M., Kuhnle G.G., Dyson A., Fernandez B.O., Grman M., DuMond J.F., Barrow M.P., McLeod G., Nakagawa H., Ondrias K., Nagy P., King S.B., Saavedra J.E., Keefer L.K., Singer M., Kelm M., Butler A.R., Feelisch M. Key bioactive reaction products of the NO/H2S interaction are S/N-hybrid species, polysulfides, and nitroxyl. Proc Natl Acad Sci

USA.2015Aug25;112(34):E4651-60.PubMedPMID:26224837.

Modulatory Role of Endogenous and Exogenous Hydrogen Sulfide on Colon Cancer Cell Proliferation: Comparison with Nitric Oxide and Carbon Monoxide

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Three gaseous molecules have been identified as gasotransmitters in the last 10 years: hydrogen sulfide (H2S), nitric oxide (NO), and carbon monoxide (CO). Each of these molecules is endogenously produced by different enzymes in various cell types and has multiple roles in normal physiology and in the pathogenesis of cancer. They also own a "bimodal" pharmacological character based on whether they have an anti- or pro-tumor effect. The three gasotransmitter systems have not yet been studied simultaneously in the same experimental system. We have studied the expression of H2S, NO and CO generating enzymes in primary colon cancer tissues and surrounding normal tissues and in the HCT116 colon cancer cell line, and investigated the effect of pharmacological inhibition and pharmacological donation of each of these transmitters on HCT116 cell proliferation. Enzyme expression was studied by Western Blotting; cell proliferation was studied using the XCelligence system. There was an increased expression of the NO synthases iNOS, eNOS, nNOS in colon cancer tissue (compared to surrounding normal tissue) and in HCT116 tumor cells (compared to the non-transformed epithelial cell line NCM356). Moreover, there was an increase in the H2S-producing enzymes CBS (cystathionine-beta-synthase) and CSE (cystathionine-gamma-lyase). In addition, there was an upregulation of heme oxygenase-1 (HO-1). Pharmacological inhibition of the H2S-producing enzymes (aminooxyacetic acid [AOAA] to inhibit CBS and CSE at 300 µM, Methyl-L-arginine [L-NMA] at 3 mM to inhibit all NOS isoforms, zinc protoporphyrin [ZnPP-IX] at 10 µM to inhibit heme oxygenase) that produce the three gasotransmitters H2S, NO and CO, as well as pharmacological donation of each of the three

gasotransmitters (using the mitochondrially targeted H2S donor AP39 at 30µM, the NO donor DETA at 100 µM and toe CO donor CORM3 at 10 µM) resulted in an inhibition of cell proliferation. These data suggest that each of the three gasotransmitters play comparable, bell-shaped roles in the control of HCT116 cell proliferation: endogenously produced low-to-mid concentrations of H2S, NO or CO support HCT116 proliferation (and, consequently, inhibition of either of them suppresses this response), while exogenous delivery of either of the gasotransmitters H2S, NO or CO (using their respective pharmacological donors) can also suppress the proliferation of the HCT116 colon cancer cells.

Standard vs off-clamp partial nephrectomy in a porcine model: Investigating the expression of hydrogen sulfide after ischemia reperfusion injury

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Centre, 339 Windermere Rd, London, Ontario, Canada N6G 2V4 Partial nephrectomy has emerged as a standard treatment option for small renal tumors (1). It has been recognized that renal function preservation impacts overall survival and controversy exists about the real functional benefit regarding zero ischemia partial nephrectomy (PN) in patients without chronic kidney disease (CKD) (2). We designed a 2/3 total nephrectomy porcine model to assess the underlying mechanisms of ischemia reperfusion injury (IRI) after PN. We evaluated intraoperative parameters, recovery of kidney function, hydrogen sulfide (H2S) levels and histological changes. Domestic male pigs (n=13) underwent left lower pole 1/3 PN and allocated to either standard (Group 1: 45 minutes of warm ischemia) or zero ischemia PN (Group 2); followed by contralateral nephrectomy. Biochemical studies were performed at baseline, day 2 and at day 7, before sacrifice. H2S was measured in urine and blood at baseline, after surgical reconstruction and on postoperative day 2 and 7 using a micro sulfide ion electrode (Lazar Research Laboratories). Preoperative characteristics between the two groups were similar. Nine pigs complete the study and those who underwent standard PN had a more torpid clinical course. Despite the significant differences in creatinine level at day 2 between the two groups (355 vs. 136 mmol/L, p=0.008), pre-sacrifice serum creatinine was similar (113 vs. 122 mmol/L, p>0.05). Blood H2S levels markedly increased after IRI and returned to baseline levels at day 7, whereas

those remained similar along the experiment in the group subjected to zero-ischemia PN. H2S levels in urine slightly increased in both groups, but return to baseline levels in animals without IRI (Table 1). The expression of pro-apoptotic markers like BAD, BAX and Caspase-3, and pro-inflammatory markers such as EDN1 and NGAL was higher in warm ischemia PN compared to off-clamp PN specimens. Despite the similar levels of creatinine between both groups before sacrifice, we found evidence of the deleterious impact of IRI during PN. Hydrogen sulfide significantly increases after IRI which may be an intrinsic protective mechanism following IRI.

Table 1. Levels of H2S in blood and urine of pigs that underwent either standard or off-clamp partial nephrectomy surgery.

	Blood H_2S (nmol/L)		Urine H_2S (nmol/L)	
	Standard	No Ischemia	Standard	No Ischemia
Baseline	1.52	1.74	0.74	1.26
Post reconstruction	1.41	1.55	3.23	2.57
Day 2	40.72	1.79	2.68	1.26
Day 7	0.72	1.45	4.09	0.88

References:

1. Huang W.C., Elkin E.B., Levey A.S. et al: Partial nephrectomy versus radical nephrectomy in patients with small renal tumors--is there a difference in mortality and cardiovascular outcomes? J Urol. 2009 Jan;(181):55.

2. Simone G., Gill I.S., Mottrie A. et al: Indications, techniques, outcomes, and limitations for minimally ischemic and off-clamp partial nephrectomy: a systematic review of the literature. Eur Urol. 2015 Oct;(68):632.

Chemical Reagents for H2S Study

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Dojindo Laboratories develops, produces and markets chemical reagents worldwide useful for life science research such as calcium ion (Ca2+) or nitric oxide (NO) signaling, and oxidative stress. Hydrogen sulfide (H2S) has emerged as the third gaseous signaling molecule along with NO and CO. The research field of H2S has been expanding with new findings of the physiological functions including vascular relaxation, celluar protection, neurotransmission and apoptosis. Furthermore, the functional mechanisms are complicating due to the emergence of new possible mediators such as persulfides, polysulfides and HNO, which should be involved in many biological systems as well as H2S and NO. Thus, it is required to develop reliable chemical reagents and methods for investigation of these reactive species separately. In addition, analyses of protein thiol modificaions such as S-sulfhydration, S-nitrosylation and S-sulfenylation, which are induced by reaction with these reactive species, will become more important in understanding of their chemical functions. Thus, we have developed chemical reagents for biological studies related to H2S, such as H2S and polysulfide donors and their detections. In our posters, the reagents which contribute to the H2S study will be introduced.

Gasotransmitter Metabolite Bioavailability during Hypoxia and Reoxygenation

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Gasotransmitters play an important role in cell signaling. Nitric oxide (NO) and hydrogen sulfide (H2S) are signaling molecules involved in regulation of cardiovascular functions including vascular remodeling as well as inflammatory responses. Their bioavailability is known to be transient and can dissipate quickly while also modifying proteins and other small molecules to alter cell signaling and function. Post-translational modifications (PTM's) influence various signaling pathways that influence pathophysiological functions associated with vascular dysfunction. However, biological fluctuations of these gasotransmitters and their impact on protein modifications during endothelial cell hypoxia and reoxygenation remain poorly understood. In this study, we examined gasotransmitter bioavailability along with possible PTM's at various time intervals during hypoxia/reoxygenation. Mouse aortic endothelial cells (MAECs) were grown to confluence and either left at normoxic (21%) conditions or placed in hypoxic (1%) oxygen tension for 30 minutes followed by 1 or 2 hour recovery in normoxic conditions. Cells were harvested in appropriate buffers and frozen in liquid nitrogen for analysis. The monobromobimane (MBB)-HPLC method was used to measure total sulfide metabolites4. Total NOx including nitrite and S-nitrosothiol (SNO) were measured using NO chemiluminescence. Free thiols were measured using a commercial fluorescent assay kit. Persulfidation/sulfhydration and s-nitrosolation were measured using tag-switch and biotin-switch methods respectively. NO bioavailability was found to decrease during hypoxia, which was not recovered by 2 hours of reoxygenation. The reduction in NO levels corresponded with increases S-nitrosylation during hypoxia and reperfusion. In contrast, total sulfide levels decreased during hypoxia with a concomitant decrease in S-sulfhydration. Interestingly, CSE protein expression was unchanged during hypoxia-reoxygenation, yet CSE enzyme activity

was significantly increased. Finally, overall levels of free thiols were decreased during hypoxia-reoxygenation. Taken together, these findings indicate that changes in sulfide bioavailability and metabolism are more acutely affected during hypoxia-reoxygenation that might be important for cellular adaptation and defense responses.

References:

1. Yuan S., Kevil C.G. Nitric Oxide and Hydrogen Sulfide Regulation of Ischemic Vascular Remodeling. Microcirculation. 2015 Sep 18.

2. Bir S.C., Pattillo C.B., Pardue S., Kolluru G.K., Docherty J., Goyette D., Dvorsky P., Kevil C.G. Nitrite anion stimulates ischemic arteriogenesis involving NO metabolism. Am J Physiol Heart Circ Physiol. 2012 Jul 15;303(2):H178-88.

3. Kolluru G.K., Yuan S., Shen X., Kevil C.G. H2S regulation of nitric oxide metabolism. Methods Enzymol. 2015;554:271-97

4. Shen X., Peter E.A., Bir S., Wang R., Kevil C.G. Analytical measurement of discrete hydrogen sulfide pools in biological specimens. Free Radic Biol Med. 2012 Jun 1-15;52(11-12):2276-83.

Dimethyl trisulfide attenuates carrageenan-induced mechanical hyperalgesia of the murine hindpaw in a TRPA1 and sst4 receptor-dependent manner

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Transient receptor potential ankyrin-1 (TRPA1) receptor-mediated activation of primary sensory neurons by dialkyl polysulfide compound dimethyl trisulfide (DMTS) was investigated. Dialkyl polysulfides might produce protein disulfides and modulate biological systems (Li and Lancaster, 2013; Gruhlke and Slusarenko, 2012). TRPA1 channels are mostly expressed in primary sensory neurons. Polysulfide compounds activate TRPA1 receptors (Kimura, 2015). TRPA1 activation might induce somatostatin (SOM) release from peptidergic sensory neurons. SOM has systemic anti-inflammatory and analgesic effects mediated by sst4 receptors (Szolcsányi et al., 2011). Ability of DMTS to release SOM and to elicit anti-inflammatory and analgesic effects via sst4 receptors in carrageenan-evoked hindpaw inflammation was examined. One hindpaw of TRPA1 and sst4 wild-type (WT) or knockout (KO) mice was injected with carrageenan (10 μL of 3% solution in saline) intraplantarly. Contralateral paws received saline. Animals were treated with DMTS (250 μmol/kg) i.p. 30 min before carrageenan challenge and every hour thereafter for 6 hours. Mechanical hyperalgesia was tested by dynamic plantar aesthesiometry. Paw oedema was detected by plethysmometry. Hyperalgesia and swelling were determined before and 2, 4, 6 h after carrageenan injection. Carrageenan induced mechanical hyperalgesia and paw swelling in all animal strains examined. DMTS significantly inhibited hyperalgesia in TRPA1 WT and sst4 WT (-50.45±2.45%, -46.49±5.59%, -55.38±2.3% vs. -13.22±4.91%, -18.62±2.17%, -18.51±3.71% and -40.69±4.05%, -51.04±3.35%, -51.33±2.42% vs. -7.29±2.91%, -20.21±4.12%, -14.78±3.02% at 2, 4, 6 h in TRPA1 and sst4 WT mice treated with vehicle or DMTS, respectively), but not in TRPA1 KO and sst4 KO

mice. Animals genetically lacking sst4 receptors developed less hyperalgesia than their WT counterparts (-51.04±3.35%, -51.33±2.42% vs. -33.34±3.27%, -30.06±1.95% at 4, 6 h in sst4 WT and KO animals treated with DMTS, respectively). Formation of hindpaw oedema was only inhibited by DMTS in TRPA1 WT mice at 6 h (66.02±3.04% vs. 37.94±6.16% in vehicle and DMTS-treated mice, respectively). Attenuating effect of repeated systemic DMTS administration on mechanical hyperalgesia induced by carrageenan is mediated by TRPA1 and sst4 receptors. DMTS might activate TRPA1 receptors on peptidergic sensory neurons and release SOM. SOM might enter the blood stream and act via sst4 receptors of nociceptor nerve endings and inflammatory cells. Inhibitory effect of DMTS on paw swelling is mediated by TRPA1 channel opening, but independent of sst4 receptors. Our data might further elucidate the pharmacology of dialkyl polysulfides and promote the development of analgesic and anti-inflammatory therapeutics of such structure.

References:

1. Gruhlke M.C., Slusarenko A.J. The biology of reactive sulfur species (RSS). Plant Physiol Biochem. 2012;59:98-107. doi: 10.1016/j.plaphy.2012.03.016.

2. Kimura H. Physiological Roles of Hydrogen Sulfide and Polysulfides. Handb Exp Pharmacol. 2015;230:61-81. doi: 10.1007/978-3-319-18144-8_3.

3. Li Q., Lancaster J.R. Jr. Chemical foundations of hydrogen sulfide biology. Nitric Oxide. 2013;35:21-34. doi: 10.1016/j.niox.2013.07.001.

4. Szolcsányi J., Pintér E., Helyes Z., Petho G. Inhibition of the function of TRPV1-expressing nociceptive sensory neurons by somatostatin 4 receptor agonism: mechanism and therapeutical implications. Curr Top Med Chem. 2011;11:2253-63.

SstR, a redox sensor from Salmonella Typhimurium regulates the sstRA operon associated with hydrogen sulfide stress tolerance

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Salmonella is an important cause of food poisoning and responsible for over a billion human infections each year (1). Following oral ingestion of Salmonella, disease is started by the bacteria being able to survive within the intestinal tract and invade epithelial cells (2). This is due, at least in part, by the ability of Salmonella to use sulfur compounds such as thiosulfate and tetrathionate for respiration thus providing a growth advantage for Salmonella and allowing it to outcompete the host microbiota to cause disease (3). However, the regulation of sulfur availability within Salmonella and the mechanisms involved in mitigating cellular hydrogen sulfide toxicity are not well-defined (4). We identified the sstRA operon in Salmonella encoding a deduced SmtB/ArsR family transcriptional regulatory protein (SstR) and deduced rhodanese-family sulfur-transferase (SstA), with a potential role in mitigating the effects of cellular hydrogen sulfide toxicity. We have confirmed that SstR acts as a repressor of transcription from the sstRA operator-promoter, with SstR-dependent repression alleviated by low pH and sulfide stress (sodium thiosulfate), consistent with a role in sulfide sensing. Electrophoretic mobility shift assays confirm binding of purified SstR to the sstRA operator-promoter. A conserved pair of cysteine residues within SstR is crucial for alleviating SstR-mediated repression, with substitution of either cysteine causing constitutive repression, consistent with SstR inducer responsiveness involving a thiol-based redox switch. Importantly, mutants lacking both sstR and sstA have reduced tolerance to sulfide stress suggesting that the sstRA operon may contribute to protecting Salmonella against cellular sulfide stress. Work is continuing to further characterize the roles of sstR and sstA in Salmonella and their contributions to infections.

References:

1. Chimalizeni Y., Kawaza K., Molyneux E. The epidemiology and management of non typhoidal salmonella infections. Advances in Experimental Medicine and Biology. 2010:33-46

2. Crump J.A., Sjolund-Karlsson M., Gordon M.A., Parry C.M. Epodemiology, Clinical Presentation, Laboratory Diagnosis, Antimicrobial Resistance and Antimicrobial Management of Invasive Salmonella Infections. Clin Microbiol Rev. 2015;28(4):901-37. doi: 10.1128/cmr.00002-15. PubMed PMID: WOS: 000368337800003.

3. Winter S.E., Baumler A.J., A breathtaking feat: to compete with the gut microbiota, Salmonella drives its host to provide a respiratory electron acceptor. Gut microbes. 2011;2(1):58-60. doi: 10.4161/gmic.2.1.14911. PubMed PMID: MEDLINE: 21637020.

4. Luebke J.L., Shen J.C., Bruce K.E., Kehl-Fie T.E., Peng H., Skaar E.P. et al. The CsoR-lile sulfurtransferase repressor (CstR) is a persulfide sensor in Staphylococcus aureus. Mol Microbiol. 2014;94(6):1343-60. doi: 10.1111/mmi.12835. PubMed PMID: WOS: 000346655900013.

S-sulfhydration in plant systems. Effect on protein subcellular location and function

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Emerging data in recent years suggest that H2S may function as an important signaling molecule in plant systems (Gotor et al., 2015; Kimura, 2015). With regard to certain stresses, H2S treatment alleviates the inhibitory effect of several abiotic stresses. H2S also plays a role in the regulation of drought stress and has been described as a component of the abscisic acid signaling network in guard cells (Scuffi et al., 2014; Papanatsiou et al., 2015). Moreover, H2S has been shown to modulate photosynthesis through the promotion of chloroplast biogenesis, photosynthetic enzyme expression, and thiol redox modification. Protein S-sulfhydration of the thiol residue of cysteines has been proposed as a mechanism for transforming the sulfide signal into a biological response and recently we have reported the detection of protein S-sulfhydryl modification in plants by using a modification of the biotin switch technique (Aroca et al., 2015). However, we have improved the detection method by using a tag-switch technique that can selectively detect persulfide adducts labelled with a biotin-linked cyanoacetate as reporting molecule (Zhang et al., 2014). With this new method, we have increased the number of detected S-sulfhydrated proteins from 106 to 3146 proteins, which are involved in functional processes mainly related with primary metabolism. We have performed a functional analysis of one of these proteins, the cytosolic isoform C of the glyceraldehyde 3-phosphate dehydrogenase enzyme, both in wild type and des1 mutant defective in the generation of sulfide in the cytosol. We have clearly observed that S-sulfhydration alters the cystosolic/nucleic subcellular location of the protein.

References:

1. Aroca Á., Serna A., Gotor C., Romero L.C. S-Sulfhydration: A Cysteine Posttranslational Modification in Plant Systems. Plant Physiol. 2015;168:334-342.

2. Gotor, C., Laureano-Marin, A.M., Moreno, I., Aroca, A., Garcia, I., Romero, L.C. Signaling in the plant cytosol: cysteine or sulfide? Amino Acids. 2015;47:2155-2164.

3. Kimura, H. Signaling of hydrogen sulfide and polysulfides. Antioxid Redox Signal. 2015;22:347-349.

4. Papanatsiou, M., Scuffi, D., Blatt, M.R., García-Mata, C. Hydrogen Sulfide Regulates Inward-Rectifying K+ Channels in Conjunction with Stomatal Closure. Plant Physiol. 2015;168:29-35.

5. Scuffi, D., Álvarez, C., Laspina, N., Gotor, C., Lamattina, L., García-Mata, C. Hydrogen Sulfide Generated by l-Cysteine Desulfhydrase Acts Upstream of Nitric Oxide to Modulate Abscisic Acid-Dependent Stomatal Closure. Plant Physiol. 2014;166:2065-2076.

4. Zhang, D., Macinkovic, I., Devarie-Baez, N.O., Pan, J., Park, C.M., Carroll, K.S., Filipovic, M.R., Xian, M. Detection of protein S-sulfhydration by a tag-switch technique. Angew Chem Int Ed Engl. 2014;53:575-581.

Transcriptional dysregulation of cysteine biosynthesis in Huntington's disease

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Regulation of amino acid homeostasis is crucial for maintenance of cellular functions. Disturbances in amino acid metabolism have been observed in Huntington's disease (HD), but their molecular origins are unknown. HD is triggered by an expansion of polyglutamine repeats in the protein huntingtin (Htt), impacting diverse cellular processes ranging from transcriptional regulation to cognitive and motor functions. We show here that the master regulator of amino acid homeostasis, activating transcription factor 4 (ATF4) is dysfunctional in HD due to oxidative stress contributed by aberrant cysteine biosynthesis and transport. Consistent with these observations, antioxidant supplementation reverses the disordered ATF4 response to nutrient stress. Our findings establish a molecular link between amino acid disposition and oxidative stress leading to cytotoxicity. This signaling cascade may be relevant to other diseases involving redox imbalance and deficits in amino acid metabolism.

References:

1. Paul B.D. et all. Cystathionine γ-lyase deficiency mediates neurodegeneration in Huntington's disease. Nature. 2014 May 1;509(7498):96-100.

2. Paul B.D., Snyder S.H. Neurodegeneration in Huntington's disease involves loss of cystathionine γ-lyase. Cell Cycle. 2014;13(16):2491-2493.

3. Paul B.D., Snyder S.H. H2S: A Novel Gasotransmitter that Signals by Sulfhydration. Trends Biochem Sci. 2015 Nov;40(11):687-700.

Synthesis of Dexamethasone-H2S Donors Hybrids and Their Evaluation in vitro

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Asthma is one of the most common chronic inflammatory disorder of the airways. Successful management of patients with severe asthma continues to be a major unmet need. One of the barriers to successful management is the heterogeneity of asthma that can be subdivided into a number of different phenotypes and endotypes. To date, the main therapies for asthma are anti-inflammatory drugs and bronchodilators, or a combination thereof. There is a growing body of evidence suggesting that hydrogen sulfide might be of clinical benefit in pulmonary diseases.[1-3] In particular a role for hydrogen sulfide in modulating airway hypereactivity and remodeling has been suggested. Airway hypereactivity is an important pathophysiological characteristic of asthma and it is linked to both airway inflammation and remodeling. Considering the biological relevance of H2S as an endogenous gasotransmitter in the pathogenesis of airway diseases,[2] and the need of new classes of drugs acting through different pathways for the unresponsive patients, we have supposed that combined treatment with glucocorticoids and H2S-releasing moieties could represent an effective therapeutic strategy for asthma care. Starting from this hypothesis we have designed and synthesized new molecular entities containing dexamethasone, chemically linked to molecules able to release H2S, that could enhance the therapeutic activity of steroids and in particular useful for steroid resistance in asthma. Dexamethasone was converted to the corresponding 21-succinate that was coupled with H2S-releasing moieties, such as ADT-OH or TBZ, leading to the formation of the desired hybrids (FAS I-II).

In order to characterize the H2S-releasing properties of the newly synthesized molecules, an amperometric approach was employed, allowing to have a real-time determination of the H2S-release and thus to perform a qualitative/quantitative description of the process. The experiments were performed at two different concentrations of the compounds (100 µM and 1 mM), in absence and in presence of 4 mM L-cysteine. The incubation of FAS I-II in the assay buffer led to a negligible release of H2S; in contrast, in the presence of L-cysteine, compounds exhibited a slow, significant and concentration-dependent release of H2S. Then, the synthesized hybrids were tested on J774 macrophages pretreated for 2h and 4h before stimulation with LPS, measuring the NO production [4] and comparing obtained data to: i) dexamethasone and ii) the corresponding succinate. Dexamethasone-H2S donors (FAS I and II) showed an enhanced inhibitory activity with respect to dexamethasone succinate when pretreated both for 2h and 4h even if the longer pretreatment resulted as the best condition with the higher % of inhibition. The obtained results supported our hypothesis that the anti-inflammatory effect could be enhanced with glucocorticoids-H2S donors hybrids, that could be suitable for the evaluation in in vivo model of asthma.

References:

1. Benetti L.R. et al. Hydrogen sulfide inhibits oxidative stress in lungs from allergic mice in vivo. Eur. J. Pharm. 2013;698:463-469.

2. Zhang G. et al. The inhibitory role of hydrogen sulfide in airway hyperresponsiveness and inflammation in a mouse model of asthma. Am J Pathol. 2013;182: 1188-1195.

3. Roviezzo F. et al. Hydrogen sulfide inhalation ameliorates allergen induced airway hypereactivity by modulating mast cell activation. Pharmacol Res. 2015;100:85-92.

4. Rossi A. Inhibition of Inducible Nitric Oxide Synthase Expression by an Acetonic Extract from Feijosellowiana Berg. Fruits. 2007;55:5053-5061.

Signaling Effects of Sodium Hydrosulfide in Healthy Donor Peripheral Blood Mononuclear Cells.

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There is increased interest in hydrogen sulfide (H2S) as an endogenous gasotransmitter in human physiology and inflammatory disease [1], however with limited knowledge of involved signal transduction pathways for this gasotransmitter in immune cells. H2S is known to modulate phosphorylation of p38 MAPK [2,3]. To examine the mechanism of action and downstream effects of p38 modulation in human PBMCs, as well as inflammatory signaling in general, we stimulated healthy donor PBMCs with sodium hydrosulfide to mimic H2S stimulation, and analysed phosphorylation of p38 MAP kinase (pT180/pY182), p-NF-κB p65 (S529), Akt (pS473) and CREB/ATF1 (pS133/pS63) with flow and mass cytometry. In contrast to subsets of lymphocytes, classical monocytes demonstrated sustained phosphorylation of p38, Akt and CREB/ATF1. NaSH induced calcium dependent phosphorylation of p38, Akt and CREB, but not NF-κB, and the phosphorylation of Akt was partly dependent on p38, indicative of p38-Akt cross talk. Akt is known to be involved in polarization of macrophages towards an anti-inflammatory M2 type, known for its involvement in resolution of inflammation [4], and such a role for H2S has been suggested by others [1]. These results

interestingly provide a description of a NaSH-induced signal transduction pathway in human primary immune cells with both pro- and anti-inflammatory aspects. This is similar to the disparate reports of the effects of H2S in inflammation [5-11].

References:

1. Wallace J.L., Wang R. Hydrogen sulfide-based therapeutics: exploiting a unique but ubiquitous gasotransmitter. Nat Rev Drug Discov. 2015;14:329-345.

2. Papapetropoulos A., Pyriochou A., Altaany Z., Yang G.D., Marazioti A., Zhou Z.M., Jeschke M.G., Branski L.K., Herndon D.N., Wang R., Szabo C. Hydrogen sulfide is an endogenous stimulator of angiogenesis. Proceedings of the National Academy of Sciences of the United States of America. 2009;106:21972-21977.

3. Rinaldi L., Gobbi G., Pambianco M., Micheloni C., Mirandola P., Vitale M. Hydrogen sulfide prevents apoptosis of human PMN via inhibition of p38 and caspase 3. Lab Invest. 2006;86:391-397.

4. Rocher C., Singla D.K. SMAD-PI3K-Akt-mTOR Pathway Mediates BMP-7 Polarization of Monocytes into M2 Macrophages. PloS one 2013;8.

5. Wallace J.L., Dicay M., McKnight W., Martin G.R. Hydrogen sulfide enhances ulcer healing in rats. Faseb J. 2007;21:4070-4076.

6. Wallace J.L., Vong L., McKnight W., Dicay M., Martin G.R. Endogenous and exogenous hydrogen sulfide promotes resolution of colitis in rats. Gastroenterology. 2009;137:569-578, 578 e561.

7. Li L., Bhatia M., Zhu Y.Z., Zhu Y.C., Ramnath R.D., Wang Z.J., Anuar F.B.M., Whiteman M., Salto-Tellez M., Moore P.K. Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. Faseb J. 2005;19:1196-1198.

8. Mok Y.Y., Atan M.S., Yoke Ping C., Zhong Jing W., Bhatia M., Moochhala S., Moore P.K. Role of hydrogen sulphide in haemorrhagic shock in the rat: protective effect of inhibitors of hydrogen sulphide biosynthesis. Br J Pharmacol. 2004;143:881-889.

9. Whiteman M., Haigh R., Tarr J.M., Gooding K.M., Shore A.C., Winyard P.G. Detection of hydrogen sulfide in plasma and knee-joint synovial fluid from rheumatoid arthritis patients: relation to clinical and laboratory measures of inflammation. Ann Ny Acad Sci. 2010;1203:146-150.

10. Ekundi-Valentim E., Santos K.T., Camargo E.A., Denadai-Souza A., Teixeira S.A., Zanoni C.I., Grant A.D., Wallace J., Muscara M.N., Costa S.K. Differing effects of exogenous and endogenous hydrogen sulphide in carrageenan-induced knee joint synovitis in the rat. Br J Pharmacol. 2010;159:1463-1474.

11. Hirata I., Naito Y., Takagi T., Mizushima K., Suzuki T., Omatsu T., Handa O., Ichikawa H., Ueda H., Yoshikawa T. Endogenous Hydrogen Sulfide Is an Anti-inflammatory Molecule in Dextran Sodium Sulfate-Induced Colitis in Mice. Digest Dis Sci. 2011;56:1379-1386.

Cystathionine-β-synthase inhibition for colon cancer: enhancement of the therapeutic efficacy of aminooxyacetic acid via the prodrug approach: *in vivo* studies

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Colon cancer cells contain high levels of cystathionine-b-synthase (CBS). Its product, hydrogen sulfide (H_2S), promotes the growth and proliferation of colorectal tumor cells *in vitro* and *in vivo* [1]. We have recently designed and synthesized an AOAA prodrug (AOAA methyl ester: YD0171) and demonstrated its superior antiproliferative effect over AOAA in various colon cancer models *in vitro*. Therefore, the efficacy of YD0171 was tested in various tumor-bearing mouse models *in vivo*. In nude mice subjected to subcutaneous or injections of HCT116 cells, animals were treated via subcutaneous vehicle, AOAA (1, 3 or 9 mg/kg/day) or YD0171 (0.1, 0.5 or 1 mg/kg/day) daily for 5 days a week for 3 weeks. Tumor growth was significantly reduced by 9 mg/kg/day AOAA, but not at the lower doses. YD0171 was more efficacious: tumor volume was significantly inhibited at 0.5 and 1 mg/kg/day. YD0171 (1 mg/kg/day) and AOAA (9 mg/kg/day) was comparably efficacious to oxaliplatin in preventing tumor growth and metastasis formation in the intracecal HCT116 tumor model. In a model of patient-derived xenograft (PDTX) in nude mice, YD0171 prevented tumor growth. In addition, delayed treatment with YD0171 (3 mg/kg/day) induced the regression of established tumors. The inhibition of CBS activity - after one week of YD0171 treatment of established tumors - was also confirmed by direct *ex vivo* measurements of CBS activity; the intratumor CBS activity inhibition was more pronounced than the inhibition of parenchymal (hepatic) CBS activity. In conclusion, the prodrug approach, as exemplified by YD0171, may be a viable strategy to increase the antitumor efficacy of AOAA.

References:

1. Szabo C., Coletta C., Chao C., Módis K., Szczesny B., Papapetropoulos A., Hellmich M.R. Tumor-derived hydrogen sulfide, produced by cystathionine-β-synthase, stimulates bioenergetics, cell proliferation, and angiogenesis in colon cancer. *Proc Natl Acad Sci U S A.* 2013;110:12474-9.

Pharmacological characterization of cardioprotective profile of the novel H_2S-donor 4-carboxyphenyl isothiocyanate (4-CPI).

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Hydrogen sulfide (H_2S) is an endogenous gasotransmitter pivotally involved in the physiological regulation of blood pressure and cardiac function. In particular, H_2S exhibits cardioprotective effects in ischemia-reperfusion (I/R) models, and is considered an important mediator of "ischemic preconditioning", a self-defence cardioprotective mechanism against myocardial I/R injury. Mechanisms of action accounting for its activity are not yet completely understood, however a central role could be played by mitochondrial ATP-sensitive potassium channels (mito-KATP), since anti-ischemic effects of H_2S are largely inhibited by mito-KATP blockers [1]. Other mechanisms are proposed to explain cardioprotection of H_2S, such as 5-phosphodiesterase inhibition and anti-inflammatory effects [2-3]. Presently, the most widely used H_2S-donor is NaHS; nevertheless this agent rapidly produces H_2S, and this feature may cause adverse effects. Indeed, for a safer and effective pharmacological administration, an ideal H_2S-donor should generate H_2S with slower releasing rate. Recently, we demonstrated the H_2S-releasing properties of some aryl isothiocyanate derivatives, suggesting that isothiocyanate is a suitable H_2S-donor moiety. Among these derivatives, 4-carboxyphenyl isothiocyanate (4-CPI) exhibited interesting concentration-dependent vasorelaxing effects on conductance and coronary arteries, and caused membrane hyperpolarization of vascular smooth muscle cells [4]. Here, we aimed

at evaluating the cardioprotective profile of 4-CPI in *ex-vivo* models of I/R injury in Langendorff-perfused hearts from Wistar rats and in *in-vivo* model of acute myocardial infarct. I/R period caused marked damage to the isolated rat hearts, highlighted by a 50% reduction in myocardial contractility; this was associated with a high degree of tissue injury, detected by morphometric analysis. 4-CPI (0.072, 0.24 and 0.72 mg/Kg, i.p.) produced a significant improvement of functional parameters and a reduced extension of ischemic area. Moreover, dihydroxyethidium (DHE)-staining evidenced an elevated production of ROS in tissue slices from hearts submitted to I/R, while in myocardial samples from rats pre-treated with 4CPI (0.24mg/Kg) ROS production was significantly reduced. Finally, CSE expression was markedly lower in cardiac homogenates obtained from hearts submitted to I/R period after pre-treated with 4CPI (0.24mg/Kg). Pre-treatment of animals with 5-hydroxydecanoic acid (5HD, 10 mg/Kg i.p.), selective blocker of mito-KATP channels, almost completely abolished the cardioprotective effects of 4CPI (0.24mg/Kg), The involvement of mito-KATP channels was further confirmed in isolated rat cardiac mitochondria, in which 4-CPI showed the typical effects of mitochondrial potassium channel activators, such as mitochondrial membrane depolarization and inhibition of calcium uptake into the mitochondrial matrix. In agreement with the results obtained on the isolated hearts, 4-CPI (0.24mg/Kg, i.p.) displayed protective effects also in *in vivo* model of acute myocardial infarct, where damaged areas were significantly reduced if compared with vehicle-treatment.

These results demonstrate that the novel H_2S-donor, 4-CPI, is endowed with significant cardioprotective activity in *ex-vivo* and *in vivo* I/R models, likely mediated by mito-KATP channel activation.

References:

1. Johansen D., Ytrehus K., Baxter G.F. Exogenous hydrogen sulfide (H2S) protects against regional myocardial ischemia-reperfusion injury—Evidence for a role of K ATP channels. Basic Res Cardiol. 2006;101:53–60.

2. Mackins C.J., Kano S., Seyedi N., Schäfer U., Reid A.C., Machida T., Silver R.B., Levi R. Cardiac mast cell-derived renin promotes local angiotensin formation, norepinephrine release, and arrhythmias in ischemia/reperfusion. J Clin Invest. 2006 Apr;116(4):1063-70.

3. Salloum F.N., Chau V.Q., Hoke N.N., Abbate A., Varma A., Ockaili R.A., Toldo S., Kukreja R.C. Phosphodiesterase-5 inhibitor, tadalafil, protects against myocardial ischemia/reperfusion through protein-kinase g-dependent generation of hydrogen sulphide. Circulation. 2009 Sep 15;120(11 Suppl):S31-6.

4. Martelli A., Testai L., Citi V., Marino A., Bellagambi F.G., Ghimenti S., Breschi M.C., Calderone V. Pharmacological characterization of the vascular effects of aryl isothiocyanates: is hydrogen sulfide the real player? Vascul Pharmacol. 2014;60:32–41.

The endothelial protective ACE inhibitor Zofenoprilat exerts anti-inflammatory activities through H_2S production

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Cardiovascular diseases as atherosclerosis are associated to an inflammatory state of the vessel wall which leads to endothelial dysfunction and adherence and activation of circulating inflammatory cells. Among the cardiovascular protecting effects of multitasking drugs as ACE inhibitors there are also their redox-reducing and anti-inflammatory properties. Hydrogen sulfide, a novel cardiovascular protective gaseous mediator, has been reported to be anti-inflammatory and we have recently demonstrated that the SH containing ACE inhibitor Zofenoprilat controls the angiogenic features of vascular endothelium though H_2S enzymatic production. Here we found that H_2S, produced through CSE activity upregulated by Zofenoprilat, abolished all the inflammatory features induced by interlukin-1beta (IL-1β) in HUVEC, especially the NF-κB/cyclooxygenase-2/prostanoid biochemical pathway. The pre-incubation with Zofenoprilat/H_2S prevented IL-1β induced paracellular iperpermeability through the control of expression and localization of cell-cell junctional markers ZO-1 and VE-cadherin. Moreover, Zofenoprilat/H_2S reduced the expression of the endothelial markers CD40 and CD31, involved in the recruitment of circulating mononuclear cells and platelets. These in vitro data document the anti-inflammatory activity of Zofenoprilat/H_2S on vascular endothelium, reinforcing the cardiovascular protective effect of this multitasking drug.

References:

1. Donnini S., Solito R., Giachetti A., Granger H.J., Ziche M., Morbidelli L. Fibroblast growth factor-2 mediates Angiotensin-converting enzyme inhibitor-induced angiogenesis in coronary endothelium. J Pharmacol Exp Ther. 2006 Nov;319(2):515-22.

2. Donnini S., Terzuoli E., Ziche M., Morbidelli L. Sulfhydryl angiotensin-converting enzyme inhibitor promotes endothelial cell survival through nitric-oxide synthase, fibroblast growth factor-2, and telomerase cross-talk. J Pharmacol Exp Ther. 2010 Mar;332(3):776-84.

3. Monti M., Terzuoli E., Ziche M., Morbidelli L. The sulphydryl containing ACE inhibitor Zofenoprilat protects coronary endothelium from Doxorubicin-induced apoptosis. Pharmacol Res. 2013 Oct;76:171-81.

4. Terzuoli E., Monti M., Vellecco V., Bucci M., Cirino G., Ziche M., Morbidelli L. Characterization of zofenoprilat as an inducer of functional angiogenesis through increased H2 S availability. Br J Pharmacol. 2015 Jun;172(12):2961-73.

Inositol polyphosphate multikinase is a regulator of transsulfuration pathway

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Inositol polyphosphate multikinase (IPMK) is one of the members of inositol phosphate kinase family that generates inositol polyphosphates. IPMK possesses inositol phosphate kinase (IP3-kinase) as well as phosphatidylinositol kinase (PI3-kinase) activities. IPMK is a pleiotropic protein and non-catalytically regulates mammalian target of rapamycin complex 1 (mTORC1), serum response factor, p300, and tumor suppressor protein p53. We report that IPMK regulates expression of cystathionine γ- lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST), enzymes involved in Hydrogen sulfide production. Protein levels of CSE and MST are increased in IPMK null fibroblasts as compared to wild type fibroblast cells. Regulation of CSE and MST is independent of catalytic activity of IPMK. IPMK regulates CSE expression at the transcriptional level. Since cystathionine β-synthase (CBS) is expressed at relatively lower levels as compare to CSE in fibroblasts, CSE is the major source of cysteine generation from cystathionine in mouse fibroblasts. Lysates prepared from IPMK null fibroblasts produced more cysteine as compared to wild type cells. Hydrogen sulfide levels are also increased in IPMK null fibroblasts. Expression of CSE is inducible upon condition such as lipopolysaccharides (LPS), and inflammation mediated by tumor necrosis factor α (TNF-α). We propose that IPMK acts as a repressor of CSE expression.

Intricate regulation of the human H2S-synthesizing enzyme cystathionine-β-synthase: the allosteric activator S-adenosyl-L-methionine elicits co and no binding

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While being involved in homocysteine homeostasis, human cystathionine β-synthase (CBS) is a major source of the gasotransmitter hydrogen sulphide (H_2S), playing a relevant role in human (patho)physiology. The CBS catalytic core domain is flanked by a C-terminal domain binding the allosteric activator S-adenosyl-L-methionine (AdoMet) and an N-terminal domain harbouring a B-type heme acting as redox sensor. AdoMet binding enhances CBS activity 2-5-fold, whereas heme reduction and CO or NO binding result in enzyme inhibition. By a combination of static and stopped-flow spectroscopy, we analysed the binding of CO and NO to the ferrous heme of recombinant human CBS and observed that NO binds >100-fold faster and >100-fold tighter than CO [1]. Moreover, whereas CO binding is rate-limited by displacement of the endogenous Cys52 ligand, NO binding is not, suggesting that NO selectively 'attacks' the heme on the His65 ligand side. We further analyzed functionally and spectroscopically the effect of AdoMet on CO and NO binding [2]. Despite being an allosteric activator of CBS, AdoMet enhances the CO inhibition of H_2S production by lowering the $K_{i(CO)}$ from 9.5 μM (AdoMet-free) to 0.7 μM (AdoMet-bound). Accordingly, AdoMet-incubated CBS binds CO ~5-fold tighter and ~10-fold faster than the AdoMet-free enzyme. Interestingly, the AdoMet effect was less

pronounced (~2-fold) on NO binding affinity and association kinetics, suggesting that the AdoMet-induced ligand modulation involves mostly the Cys52 ligand. Irrespectively of the mechanistic details entailing this communication between CBS domains, it is remarkably paradoxical that an allosteric activator makes the enzyme more prone to inactivation by the gasotransmitters CO and NO. Despite this apparent regulatory conundrum, it is not surprising that CBS, as a major source of H_2S, exhibits such intricate switching mechanisms to quickly and effectively modulate H_2S production.

References:

1. Vicente J.B., Colaço H.G., Mendes M.I., Sarti P., Leandro P., Giuffrè A. NO binds human cystathionine β-synthase quickly and tightly, J. Biol. Chem. 2014;289:8579–87.

2. Vicente J.B., Colaço H.G., Sarti P., Leandro P., Giuffrè A. S-Adenosyl-l-methionine Modulates CO and NO• Binding to the Human H2S-generating Enzyme Cystathionine β-Synthase, J. Biol. Chem. 2016;291:572–81.

AMPK Mediates the Treatment Effects of Hydrogen Sulfide and Serves as a Therapeutic Target against Anemia of Inflammation

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Aim: Anemia of inflammation (AI) is the second prevalent anemia in clinical conditions and associated with poor prognosis (1). Conventional remedies with blood transfusion and iron supplementation remain contentious due to limited effectiveness and thorny side effects. Our recent work have demonstrated that hydrogen sulfide (H_2S) markedly suppresses hepcidin, the direct cause of AI, through inhibiting interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT3) pathway (3). AMP-activated protein kinase (AMPK) acts as a metabolic master and might regulate inflammation according to recent reports (2). We thus investigated the role of AMPK in the hepcidin inhibition by H_2S and accessed the therapeutic effects of AMPK activators against AI.

Methods: Mice were pre-treated with metformin or AICAR, two AMPK activators, followed by acute (6 hours) or chronic (4 weeks) AI induction *via* injecting IL-6 or turpentine, respectively. Blood, liver, and spleen were collected and hematological indices along with body iron distribution were analyzed. For *in vitro* study, mouse primary hepatocytes were incubated with AICAR or NaHS, an H_2S donor, and then challenged with IL-6. AMPK activation by H_2S was accessed by immunoblots and AMPK kinase assay. Constrictively active AMPK (CA-AMPK), dominant negative construct (DN-AMPK) and specific AMPK siRNA were introduced to further confirm our hypothesis. Cycloheximide chase assay, ubiquitylation ladder assay, and co-immunoprecipitation were performed to examine the mechanisms in Janus kinase 2 (JAK2) degradation induced by AMPK.

Results: H_2S ameliorated IL-6-induced STAT3 phosphorylation, dimerization and transcriptional function, resulting in reduced hepcidin expression in mouse primary hepatocytes. The effects of H_2S was reversed by AMPK knockdown and a lentiviral vector expressing DN-AMPK. Moreover, activation of AMPK by AICAR and CA-AMPK suppressed inflammatory hepcidin levels as well. Similar results were obtained *in vivo*. Metformin and AICAR inhibited hepcidin production, corrected disturbances of iron homeostasis, and relieved anemic symptoms in both acute and chronic AI models. Mechanism study indicated that AMPK activation by AICAR and H_2S impaired JAK2 phosphorylation and protein levels, thus blocked IL-6-induced STAT3/hepcidin activation. Intriguingly, we found H_2S/AMPK induced JAK2 degradation in a SOCS1-dependent manner. By promoting SOCS1-JAK2 interactions, H_2S/AMPK increased JAK2 ubiquitylation, leading to proteasome-mediated degradation. Silencing and inhibition of SOCS1 diminished H_2S/AMPK-mediated suppression of STAT3 and hepcidin, as opposed to SOCS1 overexpression. Consistent results were observed *in vivo* with SOCS1 specific siRNA.

Conclusion: For the first time we demonstrate that AMPK mediates the suppression of inflammatory hepcidin by H_2S through promoting JAK2 degradation, and suggest AMPK activators as novel remedies for anemia of inflammation.

References:

1. Weiss G., Goodnough L.T. Anemia of chronic disease [J]. New England Journal of Medicine, 2005;352(10):1011-1023.

2. O'Neill L.A.J, Hardie D.G. Metabolism of inflammation limited by AMPK and pseudo-starvation [J]. Nature. 2013;493(7432): 346-355.

3. Xin H., Wang M. et all. Hydrogen sulfide attenuates inflammatory hepcidin by reducing IL-6 secretion and promoting SIRT1-mediated STAT3 deacetylation. Antioxidants & redox signaling, 2015, ahead of print.

The novel thiol-inducible H_2S donor P* triggers apoptotic cell death in Jurkat leukemia T cells

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Background/Aim: The gaseous transmitter hydrogen sulfide (H_2S) is involved in many physiological and pathophysiological processes. Numerous studies report on the role of H_2S in cell survival, proliferation and apoptosis of cancer cells but the results are controversial, depending on the type of the H_2S donor, application, etc. Here, we present a novel thiol-inducible H_2S donor, named P* [1] and described its bioactivity on Jurkat leukemia $CD4^+$ T cells.

Materials and Methods: Cell viability and apoptotic events were evaluated by Celltox Green cytotoxicity assay, Annexin-V/7-AAD staining, caspase-3/-7 assay and Western blot. Intracellular glutathione (GSH) levels were measured by GSH-Glo glutathione assay. To induce interleukin (IL)-2 expression, Jurkat T cells were stimulated with phorbol 12-myristate 13-actetate plus ionomycin. IL-2 release was quantified by enzyme-linked immunosorbent assay.

Results: Data demonstrate that P* had potent cytotoxic effects on Jurkat T cells and induced apoptotic cell death *via* the caspase-3/PARP signaling pathway. Apoptosis was completely prevented by co-treatment of the cells with sulfur-containing aminoacids like N-acetylcysteine or L-cysteine. We observed that treatment of Jurkat T cells with P* led to intracellular GSH depletion in a dose-dependent manner, suggesting that treatment with P* led to a redox imbalance, which led to apoptotic cell death. In contrast to P*, the „classic“ and fast-releasing H_2S donor sodium hydrogen sulfide did not negatively affect the viability of Jurkat T cells, suggesting that P* induced irreversible protein modifications such as formation of heat shock protein 27 dimers [2], or modification of specific cysteine residues in thioredoxin [3, and unpublished data].

References:

1. Artaud I., Galardon E. A persulfide analog of the nitrosothiol SNAP: formation, characterization and reactivity. Chembiochem. 2014 Nov 3;15(16):2361-4.

2. Suda S., Watanabe K., Tanaka Y., Watanabe K., Tanaka R., Ogihara J., Ariga T., Hosono-Fukao T., Hosono T., Seki T. Identification of molecular target of diallyl trisulfide in leukemic cells. Biosci Biotechnol Biochem. 2014;78(8):1415-7.

3. Watanabe K., Hosono T., Watanabe K., Hosono-Fukao T., Ariga T., Seki T. Diallyl trisulfide induces apoptosis in Jurkat cells by the modification of cysteine residues in thioredoxin. Biosci Biotechnol Biochem. 2014;78(8):1418-20.

A Genetic Deletion Of Cystathionine-γ-lyase Aggravates Pulmonary Inflammation And Dysfunction During Blunt Chest Trauma In Cigarette Smoke-exposed Mice

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Background: Blunt chest trauma is a common entity in polytraumatised patients and is highly associated with posttraumatic acute lung injury (ALI) [3]. The degree of pre-traumatic cigarette exposure (CS) is directly related to the severity of post-traumatic ALI [5, 7]. Since previous publications provide equivocal data regarding the role of the hydrogen sulphide (H_2S)- producing enzyme cystathionine-γ-lyase (CSE) during ALI and the development of a chronic obstructive lung disease [1, 2, 4, 6], we tested the hypothesis whether CSE deletion would aggravate pulmonary dysfunction following a blunt chest trauma in CS-exposed mice.

Materials and Methods: After 3-4 weeks of CS-exposure, anaesthetised C57BL/6 (nonCS: n=8, CS: n=8) and $CSE^{-/-}$ (nonCS: n=8, CS: n=8) mice underwent a blunt chest trauma and surgical instrumentation and immediately thereafter received lung-protective mechanical ventilation for 4 hours. Blood and lung tissue were harvested for immunoblotting, immunohistochemistry and histological evaluation.

Results: The expression of local but also systemic cytokines (interleukin (IL) 1b, IL6, IL18, TNFa) and chemokines (Keratinocyte-derived chemokine, Monocyte chemoattractant protein-1) were increased in $CSE^{-/-}$ nonCS. In line, we also verified elevated levels of oxidative stress but also pro- and anti- inflammatory mediators. Notably, local and systemic inflammation (IL1b, TNFa) was even more pronounced in $CSE^{-/-}$CS.

Conclusion: Taken together, genetic CSE deletion aggravated post-traumatic inflammation and this was further increased upon pre-traumatic CS exposure. Thus, we suggest a critical role of CSE in the post-traumatic adaptive stress response.

References:

1. Bhatia M., Wong F.L., Fu D., Lau H.Y., Moochhala S.M., Moore P.K. Role of hydrogen sulfide in acute pancreatitis and associated lung injury. FASEB J. 2005;19:623-625.

2. Chen Y.H., Yao W.Z., Geng B., Ding Y.L., Lu M. Zhao M.W., Tang C.S. Endogenous hydrogen sulfide in patients with chronic obstructive pulmonary disease. Zhonghua Jie He He Hu Xi Za Zhi. 2005;28:694-697.

3 Shah C.V., Localio A.R., Lanken P.N., Kahn J.M., Bellamy S., Gallop R., Finkel B., Gracias V.H., Fuchs B.D., Christie J.D. The impact of development of acute lung injury on hospital mortality in critically ill trauma patients. Crit.Care Med. 2008;36:2309-2315.

4. Zhou X.H., Huang X.L., Wei P., Tian F.J., Ling Y.L.: Role of hydrogen sulfide/cystathionine-gamma-lyase system in acute lung injury induced by lipopolysaccharide in rats. Zhongguo Wei Zhong Bing Ji Jiu Yi Xue. 2009;21:199-202.

5. Calfee C.S., Matthay M.A., Eisner M.D., Benowitz N., Call M., Pitter J.F., Cohen M.J. Active and passive cirgarette smoking and acute lung injury after severe blunt trauma. Am.J.Respir.Crit.Care Med. 2011;183:1660-1665.

6. Chen Y.H., Wang P.P., Wang X.M., He Y.J., Yao W.Z., Qi Y.F., Tang C.S.: Involvement of endogenous hydrogen sulfide in cigarette smoke-induced changes in airway responsiveness and inflammation of rat lung. Cytokine. 2011;53: 334-341.

7. Wagner K., Groger M., McCook O., Scheuerle A., Asfar P., Stahl B., Huber-Lang M., Ignatius A., Jung B., Duechs M., Moller P., Georgieff M., Calzia E., Radermacher P., Wagner .: Blunt Chest Trauma in Mice after Cigarette Smoke-Exposure: Effects of Mechanical Ventilation with 100% O2. PLoS One. 2015;10: e0132810.

Mitochondrial Effects Of The Novel Hydrogen Sulphide- Donor AP39

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Background: Brain, heart, kidney, and liver are organs critically affected by haemorrhagic shock due to an impaired perfusion and subsequent tissue hypoxia. A novel mitochondrially targeted hydrogen sulphide- (H_2S-) donor, AP39, has recently been shown to reveal beneficial effects in vivo by improving neurological and haemodynamic outcome in mice following cardiac arrest after pre- or early post-treatment administration [2]. Another study on rats revealed protective effects against a renal ischemia-reperfusion injury [3]. In vitro, AP39 exerts cytoprotective effects by stimulating mitochondrial respiration in rodent brain microvascular endothelial and kidney epithelial (NRK) cells [1, 3]. Therefore, we tested the effect of AP39 on four different cells lines, representing critical organs affected by shock.

Methods and measurements: AP39 (10nM and 100nM) was applied to the cell culture media of NRK cells for a time course ranging from 30min to 24h. We used two controls: AP219, which is based on a triphenylphosphonium scaffold, and ADT-OH, which serves as the actual H_2S donor. Cells were harvested for immunoblotting, mRNA

analysis and assessment of high resolution respirometry (HRR). HRR was used to measure OxPHOS-, LEAK-, and ETS-capacity (O2K Oxygraph- Oroborus Instruments, Austria). Respiratory activity of the samples was simultaneously stimulated by substrates for complex I and II (Malate, Glutamate, Pyruvat and Succinate), as well as ADP. OxPHOS-capacity was obtained as the maximum activity under all substrates including ADP; LEAK-capacity by inhibiting the ATP-synthase with Oligomycine; ETS-capacity by adding the mitochondrial chain uncoupler FCCP.
Results: AP39 showed time- and dose-dependent effects. Our data revealed reduced protein expression levels of COX subtypes (Cox4, Cox5b) following the 10nM incubation, whereas the expression was inconsistent upon 100nM incubation. AP39 further stimulated mitochondrial respiration both in the coupled and uncoupled state compared to the above mentioned controls.
Conclusion: Our data is in line with previous in vitro studies revealing an increase of mitochondrial respiration, possibly in virtue of the electron donating capacity of H_2S. The reduction of COX expression levels could be due to a delayed inhibition, thereby either preserving mitochondrial integrity or due to a ROS-mediated effect. Ongoing studies are further investigating the impact of AP39 changes upon Lipopolysaccharide (LPS)-stimulation, thereby simulating a sepsis-like state, a common post-traumatic complication. These findings are crucial for understanding the precise impact of the first mitochondrially targeted H_2S donor and its potential therapeutic role in an in vitro model of sepsis. Supported by the DFG (CRC1149, project B02).

References:

1. Szczesny B., Modis K., Yanagi K., Coletta C., Le Trionnaire S., Perry A., Wood M.E., Whiteman M., Szabo C.: AP39, a novel mitochondria-targeted hydrogen sulfide donor, stimulates cellular bioenergetics, exerts cytoprotective effects and protects against the loss of mitochondrial DNA integrity in oxidatively stressed endothelial cells in vitro. Nitric Oxide. 2014;41:120-130.

2. Ikeda K., Marutani E., Hirai S., Wood M.E., Whiteman M., Ichinose F. Mitochondria-targeted hydrogen sulfide donor AP39 improved neurological outcomes after cardiac arrest in mice. Nitric Oxide. 2015;49: 90-96.

3. Ahmad A., Olah G., Szczesny B., Wood M.E., Whiteman M., Szabo C. AP39, a mitochondrially targeted hydrogen sulfide donor, exerts protective effects in renal epithelial cells subjected to oxidative stress in vitro and in acute renal injury in vivo. Shock. 2016;45: 88-97.

Changes in the expression of hydrogen sulfide generating enzymes in murine macrophages stimulated with lipopolysaccharide and interferon-γ

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Macrophages in general, play important roles in the initiation and progression of many chronic inflammatory diseases. Lipopolysaccharide (LPS) - a potent activator of macrophages, and interferon-gamma (IFN-γ) - the principal macrophage-activating factor were used to stimulate murine macrophages J774. After stimulation J774 cells released a massive amount of pro-inflammatory mediators - TNFα and Il-6 level in culture supernatants were measured using ELISA test. Hydrogen sulfide (H_2S) plays an important role in inflammation (3). The expression of hydrogen sulfide generating enzymes i.e. cystathionine β-synthase (CBS), 3-mercaptopyruvate sulfurtransferase (MPST), and γ-cystathionase (CTH) were investigated in J774 cells. We showed that control (not stimulated) J774 cells expressed the H_2S-forming enzymes CTH and CBS and produced H_2S. The expression of the third enzyme, MPST, was not confirmed, although low activity of this enzyme was detected in J774 cells. LPS stimulated cells showed significantly increased expression of CBS and CTH in comparison to control cells. Also another study indicated increased expression of CTH in LPS stimulated cells (1). IFN-γ stimulated cells showed significantly increased expression of CTH and in a lower degree the expression of CBS. H_2S measured by the zinc acetate–trapping method (4) was used to compare H_2S level in control, and LPS and INF-g stimulated cells, after 24 and 48 h of incubation. The elevated level of H_2S was found only in cells stimulated by INF-γ after 48 h of incubation. An oxidizing environment in macrophages can result in H_2S oxidation to sulfane

sulfur. Increased level of sulfane sulfur, similarly as H_2S, was determined only in cells stimulated by INF-γ after 48 h of incubation. In LPS stimulated J774 cells both the level of H_2S and sulfane sufur were decreased after 48 h. Our results suggest that macrophages are one of the H_2S producing cells and seem to confirm the increased H_2S level in inflammation. H_2S might exert anti-inflammatory effects by inhibiting NO production (2). Changes at the molecular level not need to be parallel to the observed biochemical responses. Changes in H_2S level could be delayed to the earlier upregulation of the gene expression. H_2S can also react with nitric oxide - forms nitrosothiols in stimulated J774 cells (5).

References:

1. Li L. Bhatia M. Zhu Y.Z. Zhu Y.C. Ramnath RD. Wang ZJ. Anuar FB. Whiteman M. Salto-Tellez M. Moore PK. Hydrogen sulfide is a novel mediator of lipopolysaccharide-induce inflammation in the mouse. FASEB Journal. 2005;19:196–1198.

2. Oh G.S. Pae H.O. Lee B.S. Kim B.N. Kim J.M. Kim H.R. Jeon S.B. Jeon W.K. Chae H.J. Chung H.T. Hydrogen sulfide inhibits its nitric oxide production and nuclear factor-kB via heme oxygenase-1 expression in RAW264.7 macrophages stimulated with lipopolysaccharide. Free Radic Biol Med. 2006;41:106–119.

3. Bhatia M. Role of Hydrogen Sulfide in the Pathology of Inflammation. Scientifica 2012; 2012: 2 pages. doi:10.6064/2012/159680.

4. Kartha R.V., Zhou J., Hovde L.B., Belinda W.Y. Cheung B.W.Y. Schröder H. Enhanced detection of hydrogen sulfide generated in cell culture using an agar trap method. Analytical Biochemistry. 2012;423: 02–108.

5. Nagpure B.V. Bian J-S. Interaction of Hydrogen Sulfide with Nitric Oxide in the Cardiovascular System.Oxidative Medicine and Cellular Longevity. 2016;2016:16 pages. doi:10.1155/2016/6904327.

Resveratrol enhances endogenous H_2S-dependent relaxation in vascular tissues

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Both hydrogen sulphide (H_2S) and resveratrol (RVT) have protective effects in endothelial dysfunctions (Wang *et al.*, 2015) and cardiovascular diseases (Das *et al.*, 2007). Beside RVT and H2S share some mechanisms such as KATP and SIRT activation, PDE inhibition and ROS inhibition. We investigated if H_2S is enrolled in vascular relaxant effect of RVT in vascular tissues.We measured H_2S formation by methylene blue assay and get concentration dependent relaxations to L-cysteine and acetylcholine (ACh) by DMT myograph in aorta and corpus cavernosum. Nitric oxide synthase inhibitor Nω-Nitro-L-arginine (L-NNA), cystathionine-gamma-lyase (CSE) inhibitor PAG or cystathionine-β-synthase (CBS) and CSE inhibitor aminooxyacetic acid (AOAA) used in the presence/absence of RVT (0.1 or 0.01 mM in CC and aorta, respectively) to elucidate the role of nitric oxide (NO) or H_2S pathways on the effects of RVT. One- or Two Way Anova was used as statistical test.Vascular protective effects of RVT have been mostly attributed to eNOS activation (Frombaum et al., 2012). However we found that the ACh-induced relaxation was not altered whereas L-cysteine-induced endogenous H_2S dependent relaxations were enhanced by RVT in both aorta and CC of mice. Beside RVT stimulated both basal and L-cysteine-induced H2S formation. The augmented H_2S formation in MCC or endogenous H_2S-dependent relaxation in mice aorta did not altered by L-NNA, but returned by H_2S inhibitor AOAA or PAG, suggesting NO-independent regulation of H_2S-related effects by RVT. We concluded that RVT promotes H_2S-dependent relaxation via inducing H_2S formation regardless of tissue type. This study may be important to show the potential of H_2S in vascular protective effects of RVT and H_2S-targeting drugs in

cardiovascular diseases.

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References:

1. Das S., Das D.K. Recent Pat Cardiovasc Drug Discov. 2007;2(2):133-138.

2. Frombaum M., Le Clanche S., Bonnefont-Rousselot D., Borderie D. Biochimie. 2012;94(2):269-276.

3. Wang R., Szabo C., Ichinose F., Ahmed A., Whiteman M., Papapetropoulos A. Trends Pharmacol Sci. 2015;36(9):568-578.

L-cysteine/Hydrogen sulfide pathway is impaired under oxidative stress in mice aorta

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Oxidative stress inhibits nitric oxide (NO) bioavailability, accepted as the main cause of endothelial dysfunction and cardiovascular diseases. Several studies have been reported that hydrogen sulphide (H2S) level is impaired in endothelial dysfunctions (Wang *et al.*, 2015), where oxidative stress is accompanied. Although a recent study showed decreased MPST-induced H2S formation by H2O2 (Modis *et al.*, 2013), the role of vascular oxidative stress on the CSE/H2S - dependent regulation of ROS formation and vascular tonus has not been studied yet, as we know. We investigated the effect of pyrogallol, a ROS generator, on H2S formation by methylene blue assay and endogenous and exogenous H2S dependent relaxations by DMT strip myograph in CD1 mice aorta. Luminol and lucigenin chemiluminescences were measured as indicators of ROS formation. One way or Two way Anova was used as statistical test, when it is appropriate. We found that pyrogallol (0.1 mM, 5 min.) causes both superoxide radical and other ROS formation in mice aorta ($p<0.05$, n=7) and NaHS treatment (1 mM, 10 min.) inhibited it. ROS decreased L-cysteine stimulated endogenous H2S formation without altering basal H2S formation in mice aorta ($p<0.001$, n=7). Acute oxidative stress by pyrogallol inhibited endogenous H2S-dependent relaxation induced by L-cysteine strongly ($p<0.001$, n=6-9), while decreased ACh relaxations slightly ($p<0.05$, n=5). The impairment in L-cysteine-induced relaxation was not depending on downstream mechanism of vasorelaxation through H2S, since exogenous H2S donor NaHS-induced relaxations were not decreased by oxidative stress ($p>0.05$, n=5). We concluded that disrupted L-cysteine induced

relaxation in oxidative stress was depended on decreased endogenous H2S formation. As our knowledge this is the first study reporting impaired L-cysteine induced H2S formation and relaxation in oxidative stress. These data suggest that decreased H2S may contribute to the regulation of vascular tonus in cardiovascular disease where oxidative stress accompanies.

References:

1. Modis K., Asimakopoulou A., Coletta C., Papapetropoulos A., Szabo C. Biochem Biophys Res Commun. 2013;433(4): 401-407.

2. Wang R., Szabo C., Ichinose F., Ahmed A., Whiteman M., Papapetropoulos A. Trends Pharmacol Sci. 2015;36(9):568-578.

Hydrogen sulfide prevents vascular calcification by suppression of STAT3/Cathepsin S signaling pathway

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Arterial medial calcification (AMC), often caused by hyperglycemia and hyperphosphatemia, is one of the major causes for the higher mortality risk in the patients with diabetic renal disease. Here we studied whether hydrogen sulfide (H_2S, an endogenous gaseous mediator) can prevent aortic calcification in hyperglycemic/uremic rats. AMC was induced in rats treated with streptozotocin and subsequently fed with high fat, sugar and adenine diet for 6 weeks. At the end of the experiment, animals were sacrificed and aortic calcification was measured. Obvious calcification was observed in the aorta ofhyperglycemic/uremic rats, and this effect was significantly attenuated by treatment with NaHS (an H_2S donor, 28μmol/kg/day) for 6 weeks. H_2S also significantly reduced the contents of calcium (Ca) andphosphorus (P) and the activity of alkaline phosphatase (ALP) in the aortic tissue. It was also found that H_2S upregulated the expression of SMα-actin and SM22α, but downregulated those of osteogenic markers core binding factor alpha 1 (cbfα-1), type I collagen (CoI) and ALP. These data suggest that H2S treatment inhibits smooth muscle cells (SMCs) osteogenic switching. Moreover, H_2S reversed the phosphorylation of signal transducer and activator of transcription3 (STAT3) and the downregulated expression and activity of cathepsin S (CatS) in aorta. In addition, the accelerated degradation of elastin in hyperglycemic/uremic rats was also markedly reversed by NaHS treatment. The osteogenic phenotypic switching of SMCs is critical for the formation of AMC, we therefore treated human aortic SMCs (HASMCs) with 25 mM glucose and 10 mM β-glycerophosphate to induce SMC phenotypic transformation. It was found that both exogenous application of NaHS (100 μM) and overexpression of cystathionine γ-cystathionase (CSE, the main H_2S-producing enzyme in SMC) to increase endogenous H_2S inhibited

calcification formation in HASMCs. This effect was mimicked by a STAT3 inhibitor or a CatS inhibitor. Moreover, overexpression of CatS aggravated calcification and induced tropoelastin loss in HASMCs. On the contrary, siRNA silencing of STAT3 suppressed calcification and CatS expression in HASMCs. These results suggest that H_2S may effectively suppress high glucose and phosphate-induced SMC osteogenic phenotypic switching and calcification in a STAT3/CaS/elastin dependent manner.

Assessment of the endogenous H2S concentration *in vivo* using the mithocondria-target mass spectrometry probe MitoA

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Hydrogen sulphide (H_2S) is produced endogenously in cells and was shown to preserve mitochondrial function(1). Despite its benefits, the endogenous H_2S concentration in cells remains uncertain. The intracellular concentration of H_2S reported in the literature ranges from low nM to high µM(2, 3). The uncertainty is due in part to the lack of methods available to detect and quantify H_2S levels *in vivo*. In recent years probes for H_2S detection have been developed, the majority of which are fluorescent(4) and cannot be used in animal models *in vivo*. However, animal models are better suited to investigate pathologies like ischaemia-reperfusion injury and ethylmalonic encephalopathy, where H_2S has been implicated in the aetiology. Because of these assessed technical limitations, H_2S cannot be assessed directly in these pathologies. To address this unmet need, we developed the mitochondria-targeted mass spectrometry probe MitoA. This probe reacts selectively with free H_2S to generate a diagnostic product that can be sensitively detected by mass spectrometry, and thus can be used in animal studies to assess levels of H_2S *in vivo*. Therefore, MitoA enables the study of the variation in mitochondrial H_2S concentration *in vivo* and consequently can be used to explore the roles of H_2S in mouse models of disease.

References:

1. Elrod J.W. et al. Proc Natl Acad Sci USA.2007;104 (39): 15560-15565.

2. Furne J. et al. Am J Physiol Regul Integr Comp Physiol. 2008;295 (151):R1479-R1485.

3. Han Y. et al. Cell Mol Neurobiol. 2006;26(1):101-7.

4. Lin V.S. et al. 2015.doi: 10.1016/bs.mie.2014.11.011.

Q PerS-Sid: A Proteomic Approach for the quantitative Identification of Persulfides in Mammalian Cells

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Cysteine residues of proteins are preferred targets for modifications mediated by reactive oxygen species (ROS) and reactive nitrogen species (RNS) which can lead to dramatic changes in the stability, function, activity and localization of proteins[1]. Recently, H_2S was discovered as an important signaling molecule with high therapeutic potential in inflammation, in the cardiovascular system and in cell growth[2]. However, its exact mode of action is poorly understood. It is believed that not the gaseous form of H_2S but rather polysulfides can interact with thiols forming persulfides (R-S-SH) and thereby changing the properties of a protein[3]. However, due to its ambivalent chemical features it is hard to investigate such a modification on a protein level. On the one hand, persulfides show a similar reactivity towards electrophiles like thiols. On the other hand, they behave similar to other cysteine oxidations in a biotin switch assay. We therefore developed a mass spectrometric based method we named (q)perS-SID (quantitative persulfide site identification) which allows the specific enrichment, isolation and quantification of persulfides on a peptide level. We could show that the diverse H_2S donors Na_2S_4, Na_2S, NaSH and GYY4137 show different potencies to induce persulfides. Bioinformatical assessment of the data revealed that H_2S affects all subcellular compartments and various cellular processes. Furthermore, it seems that negatively charged amino acids appear more frequently in proximity to cysteines forming persulfides. Using PKM2 as a model protein we could confirm our proteomic data showing that persulfide formation at the cysteine residues C49, C152,

C358 and C474 leads to its inhibition. Taken together, the identification of persulfides on a proteome scale may help to better understand the biology of H_2S and explain the protective effects of H_2S in several diseases.

References:

1. Paulsen C.E., Carroll K.S. Cysteine-mediated redox signaling: chemistry, biology, and tools for discovery. Chem Rev. 2013;113(7):4633-79. doi: 10.1021/cr300163e

2. Kimura H. Hydrogen Sulfide and Polysulfides as Biological Mediators. Molecules. 2014;19,16146-16157. doi:10.3390/molecules191016146

3. Greiner. R, Pálinkás Z., Bäsell K., Becher D., Antelmann H., Nagy P., Dick T.P.Polysulfides link H2S to protein thiol oxidation. Antioxid Redox Signal. 2013;19(15):1749-65. doi: 10.1089/ars.2012.5041

Supplemental hydrogen sulfide attenuates epithelial-mesenchymal transition and mitigates renal fibrosis associated with chronic obstructive uropathy

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Introduction: Prolonged ureteral obstruction can cause fibrosis, leading to irreversible renal injury and chronic kidney disease. Recent studies suggest that transforming growth factor beta 1 (TGF-β1), a cytokine released by infiltrating inflammatory cells, can initiate epithelial-mesenchymal transition (EMT), thereby causing fibrosis (1,2). Currently, there are few preemptive measures available for mitigating renal fibrosis during obstruction. Hydrogen sulfide (H_2S) is an endogenous gasotransmitter that mediates physiological processes and can mitigate tissue injury via anti-inflammatory and anti-fibrotic properties (3–5). The current study investigates the effects of H_2S donor (GYY 4137) on renal fibrosis and EMT progression in chronic obstructive uropathy.

Methods: Male Lewis rats underwent unilateral ureteral obstruction (UUO) via ligation of left ureter. Following UUO, male Lewis rats were given daily injections of either phosphate buffered saline (PBS) or 200 μM/kg GYY 4137 + PBS for 30 days. Kidneys were removed on day 30 and stained with Masson's trichrome for histological analysis of renal fibrosis. qRT-PCR and Western blot analysis were completed on renal tissue to assess expression of EMT markers. EMT progression was also analyzed via an *in vitro* scratch wound assay

whereby a scratch wound was created with a 200μL pipette in a confluent monolayer of pig kidney epithelial cells (LLC-PK1). Cells were serum-starved for 24 hours. Cells were then treated with 10ng/mL TGF-β1 to induce EMT, as well as various doses of GYY 4137. Scratch wound was imaged every half hour for 72 hours. EMT progression was determined as a function of wound closure.

Results: Histological analysis showed significantly reduced fibrosis ($P<0.01$) in H_2S-treated rats following chronic obstruction when compared to PBS treatment. H_2S-treated rats also demonstrated significantly decreased ($P<0.05$) expression of EMT markers following chronic UUO. TGF-β1-induced progression of EMT in renal epithelial cells was significantly reduced ($P<0.05$) upon H_2S treatment.

Conclusion: Our findings suggest that supplemental H_2S can mitigate renal fibrosis associated with chronic ureteral obstruction. H_2S may represent a preemptive therapy against renal damage associated with obstructive uropathy, potentially improving long-term renal function following resolution of renal obstruction.

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References:

1. Ucero A.C., Benito-Martin A., Izquierdo M.C., Sanchez-Niño M.D., Sanz A.B., Ramos A.M. et al. Unilateral ureteral obstruction: beyond obstruction. Int Urol Nephrol. 2014;46(4):765–76.

2. Iwano M., Neilson E.G. Mechanisms of tubulointerstitial fibrosis. Curr Opin Nephrol Hypertens. 2004;13:279–84.

3. Wang R. Two’s company, three's a crowd: can H2S be the third endogenous gaseous transmitter? FASEB J. 2002 Nov;16(13):1792–8.

4. Lobb I., Sonke E., Aboalsamh G., Sener A. Hydrogen sulphide and the kidney: Important roles in renal physiology and pathogenesis and treatment of kidney injury and disease. Nitric Oxide. 2014;46(2015):55–65.

5. Lobb I., Mok A., Lan Z., Liu W., Garcia B., Sener A. Supplemental hydrogen sulphide protects transplant kidney function and prolongs recipient survival after prolonged cold ischaemia-reperfusion injury by mitigating renal graft apoptosis and inflammation. BJU Int. 2012;110(11 Pt C):E1187–95.