Different patterns of H_2S/NO activity and cross-talk in the control of the coronary vascular bed under normotensive or hypertensive conditions

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Abstract

Hydrogen sulfide (H_2S) and nitric oxide (NO) play pivotal roles in the cardiovascular system. Conflicting results have been reported about their cross-talk. This study investigated their interplays in coronary bed of normotensive (NTRs) and spontaneously hypertensive rats (SHRs).

The effects of H_2S- (NaHS) and NO-donors (sodium nitroprusside, SNP) on coronary flow (CF) were measured in Langendorff-perfused hearts of NTRs and SHRs, in the absence or in the presence of propargylglycine, (PAG, inhibitor of H_2S biosynthesis), L-NAME (inhibitor of NO biosynthesis), ODQ (inhibitor of guanylate cyclase), L-Cysteine (substrate for H_2S biosynthesis) or L-Arginine (substrate for NO biosynthesis).

In NTRs, NaHS and SNP increased CF; their effects were particularly evident in Angiotensin II (AngII)-contracted coronary arteries. The dilatory effects of NaHS were abolished by L-NAME and ODQ; conversely, PAG abolished the effects of SNP.

In SHRs, high levels of myocardial ROS production were observed. NaHS and SNP did not reduce the oxidative stress, but produced clear increases of the basal CF. In contrast, in AngII-contracted coronary arteries of SHRs, significant hyporeactivity to NaHS and SNP was observed. In SHRs, the vasodilatory effects of NaHS were only modestly affected by L-NAME and ODQ; PAG poorly influenced the effects of SNP.

Then, in NTRs, the vascular actions of H_2S required NO and vice versa. By contrast, in SHRs, the H_2S-induced actions scarcely depend on NO release; as well, the NO effects are largely H_2S-independent. These results represent a first step for understanding pathophysiological mechanisms of NO/H_2S interplays under both normotensive and hypertensive conditions.

Key words

Hydrogen sulfide, nitric oxide, coronary vessels, hypertension.

1. Introduction

Hydrogen sulfide (H_2S), a pungent gas endowed with the characteristic smell of rotten eggs, has long been recognized as a toxic agent [1,2]. Presently, its biological properties have been reconsidered, because H_2S has been recognized as an important endogenous mediator, biosynthetized in mammalian tissues from L-cysteine by cistathionine β-synthase (CBS) and cistathionine γ-lyase (CSE) [3].

In the cardiovascular system, the gasotransmitter H_2S plays a pivotal role in controlling homeostasis [4]; hence, several authors defined this mediator as "the new nitric oxide" (NO) [5]. In fact, H_2S causes relaxation of blood vessels, such as isolated rat aorta [6,7], mesenteric arteries [8] and coronary arteries [9]. In addition, recent studies have shown protective actions of H_2S against myocardial ischemia/reperfusion injury in a number of experimental models, including cultured cardiomyocytes, isolated perfused hearts and rodent models of acute myocardial infarct [10-15]. In experimental models of heart failure, H_2S increases cell survival and reduces ventricular dysfunction, thus improving heart hemodynamics and inhibiting apoptosis through mitochondrial pathways [16].

The above cardiovascular actions of H_2S are likely to be mediated by several mechanisms. Among them, opening of ATP-sensitive potassium channels (K_{ATP}) seems to play a prevalent role [3,15]. However, since H_2S-induced vasorelaxation can be blocked only in part by K_{ATP}-blockers, the involvement of additional mechanisms has been suggested [7,8]. Accordingly, the contribution of voltage-operated and calcium-activated potassium channels has been hypothesized [9,17] and, very recently, the involvement of Kv7 voltage-gated potassium of vascular smooth muscle channels has been demonstrated [18,19].

Cross-talk between NO and H_2S has been previously reported with conflicting results. Although H_2S evokes endothelium-independent vasorelaxing effects, the inhibition of endothelial NO-synthase (eNOS) or endothelium removal can attenuate the H_2S-induced vasorelaxation in rat aortic tissues [20,21]. Moreover, exogenous NO-donors trigger CSE activation in vascular smooth muscle

cells, increasing the biosynthesis of H_2S [22]. Accordingly, chronic inhibition of eNOS reduces both the expression and activity of CSE in the cardiovascular system [6,7,20].

Blunted production of endogenous H_2S or reduced CSE expression/activity can contribute to the hypertension due to NO-inhibition. Indeed, in rats with L-NAME-induced hypertension, the lack of NO biosynthesis is associated with dysfunction of the cysteine/CSE/H_2S pathway; noteworthy, the administration of exogenous H_2S prevents the development of hypertension in this model [23]. The regulation of intracellular cGMP is a further key factor accounting for a positive interaction between H_2S and NO. In one hand, NO is a potent activator of guanylate cyclase, leading to increase the biosynthesis of cGMP. In the other hand, H_2S has been reported to inhibit phosphodiesterase 5, thereby delaying the degradation of cGMP [24]. In this respect, endogenous H_2S has been reported to be essential for NO-mediated increase of cGMP, since CSE silencing abolishes the NO-induced GMPc accumulation [25].

Other authors have reported negative feedback regulations between the two gaseous mediators: on one hand, it has been hypothesized that exogenous H_2S decreases NO production, acting on the L-arginine/eNOS/NO pathway [26]. Moreover, H_2S can act as a scavenger of NO, by spontaneous chemical reactions leading to formation of an unidentified nitrosothiol. The latter mechanism might account for the NaHS-induced vasoconstriction, which has been sometimes observed in isolated vessels with intact endothelium [27,28].

Although the relaxing actions of H_2S on peripheral vasculature have been documented, its effects on coronary arteries, under healthy or pathological conditions, are largely unknown. In this study we investigated the possible interactions between H_2S and NO in this specific vascular bed in normotensive healthy rats (NTRs) and in spontaneously hypertensive rats (SHRs), a widely used animal model of genetic hypertension characterized by endothelial dysfunction associated with reduced levels of NO and excess of oxidant species [29].

2. Material and methods

Experiments were carried out, in accordance with the European Union Council Directive 86–609, on male normotensive Wistar Kyoto rats (NTRs, 300-320 g) and male SHRs (300-320 g). All the animals were housed (2 per cage) in a room under controlled temperature (23–25°C), humidity (50%) and lighting (12 h light/dark cycle), with food and water provided *ad libitum.*

On the day of experiment, the animals were euthanized with an overdose of sodium pentobarbital (100 mg/Kg, i.p.) and bled. After opening chests, hearts were quickly excised and placed in a 4 °C Krebs solution (composition mM: $NaHCO_3$ 25.0, NaCl 118.1, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2.2H_2O$ 1.6, KH_2PO_4 1.2, glucose 11.5) equilibrated with 95% plus O_2 5% CO_2, to stop the contraction and reduce oxygen consumption. Rapidly, the ascending aorta was cannulated, each heart was placed in a Langendorff apparatus and perfused (under constant pressure of 70-80 mmHg) with Krebs solution, maintained at 37 °C and bubbled continuously with gas mixture of 95% O_2 plus 5% CO_2. Then, a bolus of heparin (100 UI/100µl) was injected to prevent blood clotting. The above procedure was completed within 2 min. In order to record functional parameters, a water-filled latex balloon, connected to a pressure transducer (Bentley Trantec, mod 800), was introduced into the left ventricle through the mitral valve and the volume was adjusted to achieve a stable left ventricular end-diastolic pressure of 5-10 mmHg. Heart rate (HR) and left ventricular developed pressure (LVDP) were continuously monitored by a Biopac software (California, USA). Hearts showing severe arrhythmia or unstable LVDP and HR values, during the equilibration period, were discarded [30,31]. Coronary flow (CF) was volumetrically measured at intervals of 5 min. Raw coronary flow was expressed as ml/min, and was then normalized by the heart weight (ml/min/g). After 20-min equilibration, hearts were subjected to the subsequent experimental procedures.

The following experimental procedures were carried out on hearts from 18 groups of NTRs and 10 matched groups of SHRs (n=6 for each group). The experimental protocols and pharmacological treatments administered to each group are listed in table1. Briefly, at the end of equilibration time, hearts underwent the phase A of perfusion. The perfusion fluid employed in phase A was Krebs

solution containing L-NAME 100 μM (eNOS inhibitor), ODQ 10 μM (inhibitor of guanylate cyclase), L-Arginine 3 mM (L-Arg, substrate of eNOS), PAG 1 mM (CSE inhibitor), L-Cysteine 1 mM (L-Cys, substrate if CSE) or their vehicle. Thereafter, the phase C of perfusion was started. In this phase, a further "add-on" treatment with increasing concentrations of NaHS (H_2S-donor) or SNP (NO-donor) was carried out. Two reference vasodilators were also used in the phase C: bradykinin (BK, 10 nM-1 μM) and nifedipine (Nife, 1-100 nM). Each concentration of each vasodilator was perfused for 20 min. In order to observe the effects of the two vasorelaxing agents NaHS and SNP even in coronary vessels previously submitted to vasoconstriction, in some experimental groups, a phase B was interposed between phases A and C. During phase B, an "add-on" perfusion of Angiotensin II (AngII, 0.1 μM, a well-known effective vasoconstrictor of the coronary bed) was carried out for 20 min.

At the end of the above protocols, some hearts were submitted to hystological labeling for the determination of the oxidative stress levels.

2.1 Analysis of data

The coronary flow, recorded at the end of equilibration, was calculated as ml/min/g, and was considered as the basal value. Changes in coronary flow, following the pharmacological treatments (shown in tables 2-7), were expressed as percentage of basal coronary flow, CF (%). Moreover the net change in CF (%) induced by each vasodilator was calculated by the difference between the value of CF (%) recorded with the perfusion with the highest concentration of the vasodilator and the value of CF (%) recorded before the perfusion with the vasodilator (i.e., in the phase A for groups 1-4, 8-10 and 13-18; in the phase B for groups 5-7, 11 and 12). Data were expressed as mean ± SEM. Statistical analysis was carried out by two-way ANOVA. $P<0.05$ was considered as an indicator of significant differences.

2.2 ROS detection in myocardial tissue

For the hystological labeling and qualitative image acquisition, left ventricles from rats were isolated and readily included in Optimal Cutting Temperature (OCT, Sakura, Japan) without fixation. Cryosection 20µm thick were obtained using a manual cryostat (Leica, Germany) and collected onto glass slides.

Ventricle sections were then incubated in 10µM dihydroethidium (DHE) solution at 37°C for 30 minutes. Sections were then rinsed 3x5 minutes in Phophate buffered saline (PBS) and mounted for confocal imaging with Vectashield (Vector, Burlingame).

Confocal images were obtained for each section using a 16X oil objective (NA 0.50) on a TCS-SP2 confocal microscope (Leica, Germany). Each image was acquired as the maximum intensity projection of a 10µm confocal stack.

2.3 Drugs

AngII, BK, DHE, L-Arg, L-Cys, L-NAME, NaHS, Nife, ODQ, PAG and SNP, were purchased from Sigma-Aldrich. Sodium pentobarbital was purchased from Carlo Sessa.

3 Results

3.1 Involvement of endogenous NO in the vasoactive effects of NaHS on coronary arteries from normotensive rats

At the end of equilibration, the hearts isolated from NTRs showed a basal CF of 13.9±3.1 ml/min/g (n=108). Cumulative increasing concentrations of NaHS (1-100 µM) promoted vasorelaxation of coronary arteries, indicated by a concentration-dependent increase CF, up to a value of 126±3% (fig. 1, table 2). Perfusion of the eNOS inhibitor L-NAME (100 µM) was associated with a marked and significant CF reduction (66±1%, fig. 1). In contrast, perfusion with ODQ (10 µM) did not cause a significant reduction of the basal coronary flow (Fig. 1). However, in the presence of either

L-NAME or ODQ, NaHS almost completely lost its relaxing effects on coronary arteries (fig. 1, table 2). In the presence of high concentration of L-Arginine (3 mM), substrate of eNOS, the vasorelaxing effects of NaHS were slightly increased (fig. 1).

Perfusion with AngII (0.1μM) elicited a marked and significant reduction of CF (66±6%, fig. 2, table 3). Exposure to cumulative increasing concentrations of NaHS (1-100 μM), under the continuous presence of AngII, promoted a marked coronary vasorelaxation, indicated by the almost complete recovery of the basal flow (induced by NaHS 10 μM) and a further increase induced by NaHS 100 μM (up to 131±5%, fig. 2, table 3). Perfusion with L-NAME and the "add-on" perfusion of AngII evoked additive CF reductions, 62±1% and 44±4%, respectively. Instead, the guanylate cyclase inhibitor ODQ did not cause a further increase of the vasoconstriction induced by AngII. However, the presence of both the inhibitors of the NO-pathway almost completely prevented the vasodilator effects of NaHS in AngII-contracted coronary bed (fig. 2, table 3).

3.2 Involvement of endogenous NO in the vasoactive effects of NaHS on coronary arteries from hypertensive rats

At the end of equilibration, hearts from SHRs showed a basal CF of 11.0±1.8 ml/min/g (n=60). Basal CF in SHRs did not differ significantly from that recorded in NTRs. Cumulative increasing concentrations of NaHS (1-100 μM) promoted vasorelaxation of coronary arteries, up to a CF value of 124±7% (fig. 1, table 2). These effects were qualitatively and quantitatively equivalent to those elicited in NTRs. Perfusion with either L-NAME (100 μM) or ODQ (10 μM) did not elicit significant CF reductions (fig. 1, table 2). L-NAME significantly inhibited the effects of NaHS 1 and 10 μM; however, the highest concentration of NaHS (100 μM) evoked a significant increase (about 110±4%) in CF and almost completely restored the basal levels (fig. 1, table 2). The NaHS-induced effects were not influenced by ODQ.

Perfusion of hearts from SHRs with AngII (0.1μM) caused a marked and significant CF reduction (66±4%, fig. 2, table 3). This inhibitory effect was equivalent to that recorded in NTRs. In the

presence of AngII, the effects of NaHS were strongly reduced and only the highest concentration (100 µM) could evoke appreciable increment of CF (83±6%). In AngII-precontracted coronary arteries from SHRs, L-NAME reduced the effects of the lower concentrations of NaHS, but could not influence the vasorelaxing effects evoked by NaHS 100 µM; while the effects of the H_2S-donor concentrations were not affected at all by ODQ (fig. 2, table 3).

3.3 Involvement of endogenous H_2S in the vasoactive effects of SNP on coronary arteries from normotensive rats

Cumulative increasing concentrations of SNP (1 nM-10 µM) promoted concentration-dependent increments of CF, up to a value of 132±5% (fig. 3, table 4). Perfusion with the H_2S-biosynthesis inhibitor PAG (1 mM) did not significantly alter basal CF. In the presence of PAG, the effects of SNP were almost completely abolished (fig. 3, table 4). Conversely, in the presence of high concentration of L-Cys (1 mM), substrate for the H_2S biosynthetic pathways, the vasorelaxing effects of SNP were markedly enhanced, up to a value of 168±5%.

Perfusion with AngII (0.1µM) caused a marked and significant CF reduction (69±11%, fig. 4, table 5). Under the continuous presence of AngII, perfusion with SNP (1 nM-10 µM) elicited concentration-dependent increments of CF (up to 166±18%, fig. 4, table 5). In the presence of PAG, the "add-on" perfusion of AngII lowered CF (79±1%). The presence of PAG markedly and significantly blunted the vasodilator effects of SNP in AngII-contracted coronary bed (fig. 4, table 5).

3.4 Involvement of endogenous H_2S in the vasoactive effects of SNP on coronary arteries from hypertensive rats

Cumulative increasing concentrations of SNP (1 nM-10 µM) promoted a concentration-dependent increase in the CF, up to a value of 133±6% (fig. 3; table 4). Perfusion with PAG (1 mM) did not

significantly alter the basal CF. PAG attenuated (but did not abolish) the effects of SNP (fig. 3, table 4).

Perfusion with AngII (0.1μM) evoked a marked and significant reduction of CF (77±5%, fig. 4, table 5). SNP (1 nM-10 μM) under the continuous presence of AngII, promoted concentration-dependent increments of CF (up to 125±5%, fig. 4, table 5). In the presence of PAG, the "add-on" perfusion of AngII lowered CF (64±7%). The presence of PAG significantly reduced (but did not abolish) the effects of SNP on AngII-contracted coronary vessels (fig. 4, table 5).

3.5 Involvement of endogenous NO and H_2S in the vasoactive effects of bradykinin on coronary arteries from normotensive rats

Cumulative increasing concentrations of BK (10 nM - 1 μM) promoted marked vasorelaxation of coronary arteries, indicated by a concentration-dependent increase of CF, up to a value of 173±4%. The eNOS inhibitor L-NAME (100 μM) caused significant CF reduction (72±9%, fig. 1), while the CSE inhibitor PAG did not influence the basal CF. However, both the enzyme inhibitors almost completely abolished the vasorelaxing effects of BK (fig. 5A).

3.6 Involvement of endogenous NO and H_2S in the vasoactive effects of nifedipine on coronary arteries from normotensive rats

Cumulative increasing concentrations of Nife (1 nM - 100 nM) promoted concentration-dependent increases of CF, up to a value of 147±8%. L-NAME (100 μM) caused significant CF reduction (60±2%, fig. 1), while the CSE inhibitor PAG did not influence the basal CF. Preincubation with both the inhibitors did not blunt the vasorelaxing activity of Nife (fig. 5B).

3.7 Effects of NaHS and SNP on the ROS production in myocardial tissue of normotensive and hypertensive rats

The DHE staining of the myocardial tissue of NTRs evidenced a very low level of ROS production. In contrast, the hearts of SHRs exhibited a high density of DHE-stained nuclei, indicating a significant level of oxidative stress and ROS production. The acute treatment with either NaHS or SNP did not reduce the level of DHE-staining in myocardial samples of SHRs (fig. 6).

4. Discussion

It is presently known that the H_2S pathway is essential in regulating the circulation homeostasis, through a number of different mechanisms and sites of action. Among them, the vasorelaxing effect is likely to be pivotal for fine tuning of specific vascular beds, such as coronary arteries [3]. Consistently, in the present study, the H_2S-releasing agent NaHS elicited concentration-dependent increments of basal CF. Of note, these effects were similar in hearts from NTRs and SHRs. AngII evoked equivalent vasoconstriction in the coronary bed of both NTRs and SHRs, as indicated by comparable reductions of CF in both the experimental groups. When applied to AngII-constricted coronary vessels of NTRs, NaHS evoked marked relaxing effects. In contrast, the NaHS-induced vasorelaxation was significantly reduced (albeit not abolished) in AngII-constricted coronary vessels from SHRs, indicating that the AngII-vasoconstricted coronary bed of SHRs is hyporesponsive to NaHS, with respect to the vasoconstricted coronary bed of NTRs.

Perfusion with the NO-synthase inhibitor L-NAME was associated with a remarkable constriction of coronary arteries from NTRs, reflected by a drastic fall of CF, indicating that endothelial NO markedly contributes to the modulation of coronary smooth muscle tone.

Notably, the guanylate cyclase inhibitor ODQ caused negligible reduction of the CF. The above different effects of L-NAME and ODQ are likely to be due to the specific mechanisms of action of the two inhibitors. In fact, although cGMP is the main effector of the relaxant response to NO, it is widely accepted that additional cGMP-independent pathways exist [32]. Of course, L-NAME, inhibitor of NO biosynthesis, suppresses all the NO-activated vasodilator pathways, thus producing more dramatic effects. While ODQ, inhibitor of the guanylate cyclase, can affect only one of the

NO-mediated pathways. Interestingly, in NTRs, the effects of NaHS were completely abolished by both L-NAME and ODQ. Therefore, under normotensive conditions, both the H_2S and NO pathways play significant roles in the control of CF, and the relaxing action of H_2S on coronary vessels largely requires NO-mediated cooperation. Although previous papers reported that the H_2S-induced relaxation of coronary bed is endothelium-independent and cannot be affected by eNOS inhibition [9], it should be remarked that those studies were not carried out by evaluating CF in isolated hearts, but by myographic recordings from isolated coronary arteries. Thus, methodological issues are likely to account for this discrepancy. Moreover, our evidence is consistent with other reports, showing clear synergic activity between the two gasotransmitters [20,21].

In hearts from SHRs, basal CF was similar to that recorded in NTRs. Nevertheless, the L-NAME-induced CF decrement, although significantly evident, was markedly lower than that recorded in hearts from NTRs, thus providing evidence that endogenous endothelial NO only in part contributes to the modulation of vasomotor tone in coronary smooth muscle from SHRs, as already documented by other authors [33]. It is also noteworthy that the influence of L-NAME and ODQ on the dilatory effects exerted by NaHS in hypertensive coronary arteries was significantly different from those observed in normotensive vessels. L-NAME and ODQ (which completely abolished the effects of NaHS in NTRs) very modestly attenuated the effects of NaHS in vessels from SHRs.

In the present study, the NO-releasing agent SNP evoked almost equivalent concentration-dependent increments of basal CF, in both NTRs and SHRs. When applied to AngII-constricted coronary vessels of NTRs, SNP still produced marked vasorelaxing effects. In contrast, in AngII - constricted coronary bed of SHRs, the effects of SNP were significantly reduced. Therefore, as above observed for the vasorelaxing effects of NaHS, the vasoconstricted coronary bed of SHRs is hyporesponsive even to SNP. Perfusion with the CSE-inhibitor PAG resulted in a very modest (not significant) decrease in the CF of SHRs, while no effect at all was recorded in normotensive coronary arteries. This observation might indicate the possible existence of small difference in the modulatory role of endogenous H_2S, but this hypothesis will be addressed by more specific studies.

Pre-treatment of normotensive coronary vessels with the CSE inhibitor PAG, led to an almost complete abolition of the effects of SNP on basal CF. Conversely, pre-treatment of normotensive coronary vessels with the CSE substrate L-Cys, led to a significant increase in the vasorelaxing effects evoked by SNP on the CF. In contrast, PAG caused a very modest influence on the effects of SNP in the coronary bed of SHRs. Analogously, in AngII-contracted vessels form NTRs, the relaxing effects of SNP were strongly inhibited by PAG; while, in AngII-contracted vessels from SHRs, the vasodilating effects of SNP were poorly influenced by the CSE-inhibitor.

In order to strengthen the above observation, further experiments were carried out with BK, a vasorelaxing peptide, whose effects are largely mediated by the release of endothelial NO [34-36]. As expected, the coronary vasodilator response to BK was completely abolished by L-NAME. Notably, even PAG drastically inhibited the vasorelaxing activity of bradykinin. In contrast, the effects of Nife, a well-known endothelium-independent vasodilator, were not influenced by L-NAME and PAG.

The results of these experiments point out the interesting concept that, under normotensive conditions, the positive control of the NO pathway on CF is strongly dependent on endogenous H_2S; while, under hypertensive conditions, such a H_2S-dependence undergoes a significant attenuation. Hence, a strong and "specular" analogy between the two gasotransmitters has been clearly observed: in the coronary arteries of NTRs, the vasorelaxing effects induced by either H_2S or NO seem to obligatorily require a mutual interaction. H_2S and NO have very complicated and sometime conflicting relationships, which can rely in direct chemical interplay and/or in the reciprocal regulation of biosynthesis. For instance, the expression and activity of CSE in vascular smooth muscle cells are increased by NO releasing agents [6]; as well, eNOS activation by H_2S through a p38 MAPK and Akt-dependent pathway has been recently reported [37]. Furthermore, the activation of K_{ATP} channels, expressed in the vascular smooth muscle cells, is a well documented pharmacological mechanism of H_2S [7]. Since it has been reported that the activation of endothelial K_{ATP} channels is followed by the release of NO [38], the activation of these ion channel in the

endothelial cells by H_2S may be a further mechanism of NO/H_2S interaction. Even the role of downstream effectors, such as the cyclic nucleotides, must be greatly taken into account [39,40].

Although a mechanistic investigation of the H_2S/NO interplay, observed in rat coronary bed, has not been specifically carried out in this study, the strong inhibition evoked by ODQ on the H_2S-mediated effects in normotensive conditions strongly suggest that such an interaction may largely reside in the generation/preservation of intracellular cGMP levels, as also observed by other authors. Indeed, the effects of the two gasotransmitters converge on cGMP: NO directly activate soluble guanylyl cyclase, while H_2S has been reported to maintain a tonic inhibitory effect on PDE5, thus delaying the degradation of cGMP [24,25].

In the other hand, in the setting of hypertension, the relaxing action of H_2S on coronary vessels appears to scarcely depend on NO; as well, the vasodilator effects of NO are quite independent from H_2S. The present work cannot give an exhaustive explanation for this hypertension-associated change in the H_2S/NO interaction; however, it should be pointed out that the myocardial tissue of SHRs showed a highest level of oxidative stress, evidenced by large production of ROS, as already described [41]. Although it has been reported that the oxidative stress is abolished by chronic treatment with NaHS [41], in our experimental conditions (i.e. acute treatment), neither NaHS nor SNP could reduce the excessive ROS production in the hearts of SHRs. It is well known that such an excessive ROS generation promotes a significant decrease in bioavailability of NO [29, 42, 43]. Accordingly, since H_2S is a strong reducing agent which avidly scavenges ROS and analogous reactive compounds [44,45], a highest concentration of ROS can actually impair the concentrations of endogenous H_2S. Such a reduced bioavailability of NO and H_2S may have triggered, as an adaptive response, the strengthening of H_2S- and NO-independent mechanisms, respectively, for the vasorelaxing effects of the two gasotransmitters.

5. Conclusions

Our findings demonstrate, for the first time, the existence of different regulatory patterns in the H_2S/NO interplays in the coronary arteries of normotensive and hypertensive animals. In particular, in NTRs, the coronary relaxing actions of H_2S appear to obligatorily require NO and, vice versa, the relaxing actions of NO require H_2S. On the basis of previous reports, describing the H_2S-mediated inhibition of phosphodiesterase 5 in vascular smooth muscle [24], it can be hypothesized that, under normotensive conditions, H_2S and NO can mutually interact converging into an intracellular GMPc accumulation. This hypothesis agrees with other reports, showing that endogenous H_2S is essential for the NO-mediated cGMP increase, and that the suppression of CSE abolishes the NO-induced cGMP accumulation [25]. In contrast, such a mutual interplay between NO and H_2S pathways is lost in SHRs. This would indicate that the hypertensive status leads to unmask (or to strengthen) both NO-independent mechanisms for H_2S, and H_2S-independent mechanisms for NO.

Overall, the present findings represent a first important step for understanding the pathophysiological mechanisms accounting for the complex NO/H_2S regulatory interplay both under normotensive and hypertensive conditions. Moreover, our data will help to identify and optimize novel pharmacological strategies focused on the H_2S and/or NO pathways, for the management of coronary diseases.

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Conflict of interest

The authors declare no conflict of interest.

References

[1] C.V. Winder, H.O. Winder. The seat of action of sulfide on pulmonary ventilation, Am. J. Physiol. 105 (1933) 337-352.

[2] R.P. Smith, R.E. Gosselin. Hydrogen sulphide poisoning, J. Occup. Med. 21 (1979) 93-97.

[3] A. Martelli, L. Testai, M.C. Breschi, C. Blandizzi, A. Virdis, S. Taddei, V. Calderone. Hydrogen sulphide: novel opportunity for drug discovery, Med. Res. Reviews 32 (2012) 1093-1130.

[4] D.J. Lefer. A new gaseous signaling molecule emerges: Cardioprotective role of hydrogen sulfide, Proc. Natl. Acad. Sci. USA. 46 (2007) 17907-17908.

[5] K. Sanderson. Physiology: Emissions control, Nature 459 (2009) 500–502.

[6] R. Hosoki, N. Matsuki, H. Kimura. The possible role of hydrogen sulphide as an endogenous smooth muscle relaxant in synergy with nitric oxide, Biochem. Biophys. Res. Commun. 237 (1997) 527-531.

[7] W. Zhao, J. Zhang, R. Wang. The vasorelaxant effect of H2S as a novel endogenous gaseous KATP channel opener, EMBO. J. 20 (2001) 6008-6016.

[8] Y. Cheng, J.F. Ndisang, G. Tang, K.K. Cao, R. Wang. Hydrogen sulfide-induced relaxation of resistance mesenteric artery beds of rats, Am. J. Physiol. Heart Circ. Physiol. 287 (2004) H2316-2323.

[9] W.S. Cheang, W.T. Wong, B. Shen, C.W. Lau, X.Y. Tian, S.Y. Tsang, X. Yao, Z.Y. Chen, Y. Huang. 4-aminopyridine-sensitive K+ channels contributes to NaHS-induced membrane hyperpolarization and relaxation in the rat coronary artery, Vasc. Pharmacol. 53 (2010) 94-98.

[10] J.S. Bian, Q.C. Yong, Y.Y. Mok, L. Ling, M. Whiteman, M. Bhatia, P.K. Moore. Role of hydrogen sulfide in the cardiprotection caused by ischemic preconditioning in the rat heart of cardiac myocytes, J. Pharmacol. Exp. Ther. 316 (2006) 670-678.

[11] J.W. Elrod, J.W. Calvert, J. Morrison, J.E. Doeller, D.W. Kraus, L. Tao, X. Jiao, R. Scalia, L. Kiss, C. Szabo, H. Kimura, C.W. Chow, D.J. Lefer. Hydrogen sulfide attenuates myocardial

ischemia-reperfusion injury by preservation of mitochondrial function, Proc. Natl. Acad. Sci. USA 104 (2007) 15560-15565.

[12] H. Wei, R. Zhang, H. Jin, X. Liu, C. Tang, J. Du. Hydrogen sulfide attenuates hyperhomocysteinemia-induced cardiomyocytic endoplasmic reticulum stress in rats, Antioxid. Redox Signal. 12 (2010) 1079-1091.

[13] L.L. Yao, X.W. Huang, Y.G. Wang, Y.X. Cao, C.C. Zhang, Y.C. Zhu. Hydrogen sulphide protects cardiomyocytes from hypoxia/reoxygenation-induced apoptosis by preventing GSK-3beta-dependent opening of mPTP, Am. J. Physiol. Heart Circ. Physiol. 298 (2010) H1310-H1319.

[14] Y. Ji, Q.F. Pang, G. Xu, L. Wang, L.K. Wang, Y.M. Zeng. Exogenous hydrogen sulfide postconditioning protects isolated rat hearts against ischemia-reperfusion injury, Eur. J. Pharmacol. 587 (2008) 1-7.

[15] Z. Zhang, H. Huang, P. Liu, C. Tang, J. Wang. Hydrogen sulfide contributes to cardioprotection during ischemia-reperfusion injury by opening KATP channels, Can. J. Physiol. Pharmacol. 85 (2007) 1248-1253.

[16] X. Wang, Q. Wang, W. Guo, Y.Z. Zhu. Hydrogen sulfide attenuates cardiac dysfunction in a rat model of heart failure; a mechanism through cardiac mitochondrial protection, Biosci. Rep. 31 (2011) 87-98.

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

[18] A.K. Mustafa, G. Sikka, S.K. Gazi, J. Steppan, S.M. Jung, A.K. Bhunia, V.M. Barodka, F.K. Gazi, R.K. Barrow, R. Wang, L.M. Amzel, D.E. Berkowitz, S.H. Snyder. Hydrogen sulfide as endothelium-derived hyperpolarizing factor sulfhydrates potassium channels, Circ. Res. 109 (2011) 1259-1268.

[19] M. Gollasch. Vasodilator signals from perivascular adipose tissue, Br. J. Pharmacol. 165 (2012) 633-642.

[20] W. Zhao, J.F. Ndisang, R. Wang. Modulation of endogenous production of H2S in rat tissues, Can. J. Physiol. Pharmacol. 81 (2003) 848-853.

[21] W. Zhao, R. Wang. H2S-induced vasorelaxation and underlying cellular and molecular mechanisms, Am. J. Physiol. Heart Circ. Physiol. 283 (2002) H474-H480.

[22] W. Zhao, J.F. Ndisang, R. Wang. Modulation of endogenous production of H_2S in rat tissues, Can. J. Physiol. Pharmacol. 81 (2003) 848–853.

[23] G. Zhong, F. Chen, Y. Cheng, C. Tang, J. Du. The role of hydrogen sulfide generation in the pathogenesis of hypertension in rats induced by inhibition of nitric oxide synthase, J. Hypertens. 21 (2003) 1879-1885.

[24] M. Bucci, A. Papapetropoulos, V. Vellecco, Z. Zhou, A. Pyriochou, C. Roussos, F. Roviezzo, V. Brancaleone, G. Cirino. Hydrogen sulfide is an endogenous inhibitor of phosphodiesterase activity, Arterioscler. Thromb. Vasc. Biol.30 (2011) 1998-2004.

[25] C. Coletta, A. Papapetropoulos, K. Erdelyi, G. Olah, K. Modis, P. Panopoulos, A. Asimakapoulou, D. Gero, I. Sharina, E. Martin, C. Szabo. Hydrogen sulfide and nitric oxide are mutually dependent in the regulation of the angiogenesis and endothelium-dependent vasorelaxation, Proc. Natl. Acad. Sci. USA 109 (2012) 9161-9166.

[26] B. Geng, Y. Cui, J. Zhao, F. Yu, Y. Zhu, G. Xu, Z. Zhang, C. Tang, J. Du. Hydrogen sulfide downregulates the aortic L-arginine/nitric oxide pathway in rats, Am. J. Physiol. Regul. Integr. Comp. Physiol. 293 (2007) R1608-R1688.

[27] M. Whiteman, L. Ling, I. Kostetski, S.H. Chu, J.L. Siau, M. Bhatia, P.K. Moore. Evidence for the formation of a novel nitrosothiol from the gaseous mediators nitric and hydrogen sulphide, Biochem. Biophys. Res. Commun. 343 (2006) 303-310.

[28] M.Y. Ali, C.Y. Ping, Y.P. Mok, M. Whiteman, M. Bhatia, P.K. Moore. Regulation of vascular nitric oxide in vitro and in vivo; role for endogenous hydrogen sulphide?, Br. J. Pharmacol. 149 (2006) 625-634.

[29] A. Virdis, R. Colucci, D. Versari, N. Ghisu, M. Fornai, L. Antonioli, E. Duranti, E. Daghini, C. Giannarelli, C. Blandizzi, S. Taddei, M. Del Tacca. Atorvastatin prevents endothelial dysfunction in mesenteric arteries from spontaneously hypertensive rats: role of cyclooxygenase 2-derived contracting prostanoids, Hypertension. 53 (2009) 1008-1016.

[30] M.C. Breschi, V. Calderone, M. Digiacomo, M. Manganaro, A. Martelli, F. Minutolo, S. Rapposelli, L. Testai, F. Tonelli, A. Balsamo. Spirocyclic benzopyran-based derivatives as new anti-ischemic activators of mitochondrial ATP-sensitive potassium channel, J. Med. Chem. 51 (2008) 6945-6954.

[31] L. Testai, A. Martelli, A. Marino, V. D'Antongiovanni, F. Ciregia, L. Giusti, A. Lucacchini, M.C. Breschi, V. Calderone. The activation of mitochondrial BK potassium channels contributes to the protective effects of naringenin against myocardial ischemia/reperfusion injury, Biochem. Pharmacol. 85 (2013) 1634-1643.

[32] J.C. Wanstall, K.L. Homer, S.A. Doggrell. Evidence for, and importance of, cGMP-independent mechanisms with NO and NO donors on blood vessels and platelets, Curr. Vasc. Pharmacol. 3 (2005) 41-53.

[33] E. Moltzer, A.V. Verkuil, R. van Veghel, A.H. Danser, J.H. van Esch. Effects of angiotensin metabolites in the coronary vascular bed of the spontaneously hypertensive rat: loss of angiotensin II type 2 receptor-mediated vasodilation, Hypertension 55 (2010) 516-522.

[34] K.P.J. O'Kane, D.J. Webb, J.G. Collier, P.J.T. Vallance. Local L-NG-monomethyl-arginin]e attenuates the vasodilator action of bradykinin in the human forearm, Br. J. Clin. Pharmacol. 38 (1994) 311–315.

[35] J.V. Mombouli, S. Illiano, T. Nagao, T. Scott-Burden, P.M. Vanhoutte. Potentiation of endothelium-dependent relaxations to bradykinin by angiotensin I converting enzyme inhibitors in canine coronary artery involves both endothelium-derived relaxing and hyperpolarizing factors, Circ. Res. 72 (1992) 137–144.

[36] T. Nagao, S. Illiano, P.M. Vanhoutte. Heterogeneous distribution of endothelium-dependent relaxations resistant to NG-nitro-L-arginine in rats, Am. J. Physiol. 263 (1992) H1090–H1094.

[37] Z. Altaany, G. Yang, R. Wang. Crosstalk between hydrogen sulfide and nitric oxide in endothelial cells, J. Cell. Mol. Med. 17 (2013) 879-888.

[38] M.E. Gendron, E. Thorin, L.P. Perrault. Loss of endothelial K_{ATP} channel-dependent, NO-mediated dilation of endocardial resistance coronary arteries in pigs with left ventricular hypertrophy, Br. J. Pharmacol. 143 (2004) 285-291.

[39] S.I. Bibli, G. Yang, Z. Zhou, R. Wang, S. Topouzis, A. Papapetropoulos. Role of cGMP in hydrogen sulfide signaling, Nitric Oxide. 14 (2014) S1089-S8603.

[40] M. Bucci, A. Papapetropoulos, V. Vellecco, Z. Zhou, A. Zaid, P. Giannogonas, A. Cantalupo, S. Dhayade, K.P. Karalis, R. Wang, R. Feil, G. Cirino. cGMP-dependent protein kinase contributes to hydrogen sulfide-stimulated vasorelaxation, PLoS ONE 7 (2012) e53319 doi: 10.1371/journal.pone.0053319.

[41] Y.X. Shi, Y. Chen, Y.Z. Zhu, G.Y. Huang, P.K. Moore, S.H. Huang, T. Yao, Y.C. Zhu. Chronic sodium hydrosulfide treatment decreases medial thickening of intramyocardial coronary arterioles, interstitial fibrosis, and ROS production in spontaneously hypertensive rats, Am. J Physiol. Heart Circ. Physiol. 293 (2007) H2093-H2100.

[42] H. Cai, D.G. Harrison. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress, Circ. Res. 87 (2000) 840-844.

[43] H.J. Hsieh, C.A. Liu, B. Huang, A.H. Tseng, D.L. Wang. Shear-induced endothelial mechanotransduction: the interplay between reactive oxygen species (ROS) and nitric oxide (NO) and the pathophysiological implications, J. Biomed. Sci. 21 (2014) 3. doi: 10.1186/1423-0127-21-3.

[44] B. Geng, L. Chang, C. Pan, Y. Qi, J. Zhao, Y. Pang, J. Du, C. Tang. Endogenous hydrogen sulfide regulation of myocardial injury induced by isoproterenol, Biochem. Biophys. Res. Commun. 318 (2004) 756-763.

[45] L. Chang, B. Geng, F. Yu, J. Zhao, H. Jiang, J. Du, C. Tang. Hydrogen sulfide inhibits myocardial injury induced by homocysteine in rats, Amino Acids. 34 (2008) 573-585.

Legends

Figure 1 - The bars indicate the net changes from the basal CF (%) induced by the whole cumulative administration of NaHS, in heart of normotensive (NTR) or hypertensive (SHR) animals, in the presence of the indicated enzyme inhibitors or substrates, or of the corresponding vehicle. Vertical bars represent standard errors (n=6).

Figure 2 - The bars indicate the net changes from the AngII-reduced CF (%) induced by the whole cumulative administration of NaHS, in heart of normotensive (NTR) or hypertensive (SHR) animals, in the presence of the indicated enzyme inhibitors, or of the corresponding vehicle. Vertical bars represent standard errors (n=6).

Figure 3 The bars indicate the net changes from the basal CF (%) induced by the whole cumulative administration of SNP, in heart of normotensive (NTR) or hypertensive (SHR) animals, in the presence of the indicated enzyme inhibitors or substrates, or of the corresponding vehicle. Vertical bars represent standard errors (n=6).

Figure 4 The bars indicate the net changes from the AngII-reduced CF (%) induced by the whole cumulative administration of SNP, in heart of normotensive (NTR) or hypertensive (SHR) animals, in the presence of the indicated enzyme inhibitors, or of the corresponding vehicle. Vertical bars represent standard errors (n=6).

Figure 5 The bars indicate the net changes from the AngII-reduced CF (%) induced by the whole cumulative administration of BK (A) or Nife (B), in heart of NTRs, in the presence of the indicated enzyme inhibitors, or of the corresponding vehicle. Vertical bars represent standard errors (n=6).

Figure 6 Confocal images of ventricle sections from normotensive and hypertensive rat hearts perfused with vehicle (NTRs and SHRs, respectively), and of hypertensive rat hearts perfused with NaHS (SHR/NaHS) or SNP (SHR/SNP). Oxidative stress in the tissue was revealed by DHE staining. Right columns represent magnification of the boxed areas. Arrows indicate cells generating superoxide radicals in response to oxidative stress.

Table 1 - Pharmacological treatments applied in phase A, B and C, for the 18 groups of normotensive animals (groups 1n-18n) and the 10 groups of hypertensive animals (groups 1h-3h, 5h-9h, 11h and 12h). Phase B was not carried out for the groups 1-4, 8-10 and 13-18.

	Phase A	**Phase B**	**Phase C**
GROUP 1n / GROUP 1h	vehicle	-	NaHS (1-100µM)
GROUP 2n / GROUP 2h	L-NAME (100µM)	-	NaHS (1-100µM)
GROUP 3n / GROUP 3h	ODQ	-	NaHS (1-100µM)
GROUP 4n	L-Arg	-	NaHS (1-100µM)
GROUP 5n / GROUP 5h	vehicle	AngII (0.1µM)	NaHS (1-100µM)
GROUP 6n / GROUP 6h	L-NAME (100µM)	AngII (0.1µM)	NaHS (1-100µM)
GROUP 7n / GROUP 7h	ODQ	AngII (0.1µM)	NaHS (1-100µM)
GROUP 8n / GROUP 8h	vehicle	-	SNP (1nM-10µM)
GROUP 9n / GROUP 9h	PAG (1mM)	-	SNP (1nM-10µM)
GROUP 10n	L-Cys	-	SNP (1nM-10µM)
GROUP 11n / GROUP 11h	vehicle	AngII (0.1µM)	SNP (1nM-10µM)
GROUP 12n / GROUP 12h	PAG (1mM)	AngII (0.1µM)	SNP (1nM-10µM)
GROUP 13n	vehicle	-	BK (10nM-1µM)
GROUP 14n	L-NAME (100µM)	-	BK (10nM-1µM)
GROUP 15n	PAG (1mM)	-	BK (10nM-1µM)
GROUP 16n	vehicle	-	Nife (1-100nM)
GROUP 17n	L-NAME (100µM)	-	Nife (1-100nM)
GROUP 18n	PAG (1mM)	-	Nife (1-100nM)

Table 2 - Changes in CF (% of basal value) recorded during the perfusion of hearts isolated from NTRs (upper) and SHRs (lower) with cumulative concentrations of NaHS (phase C, 1-100μM), in the experimental groups 1n-4n and 1h-3h.
([a]) The pharmacological treatments with L-NAME (or the corresponding vehicle) added in phase A are indicated in parentheses.
Statistically different from the corresponding value recorded in Phase A (= P<0.05; ** = P<0.01; *** = P<0.001).
[#] Statistically different from the corresponding basal value recorded in vehicle-treated hearts ([##] = P<0.01; [###] = P<0.001).

	NORMOTENSIVE			
	Phase A	*Phase C*		
	([a])	*NaHS 1 μM*	*NaHS 10 μM*	*NaHS 100 μM*
Group 1n (vehicle)	100±1	116±3***	119±4***	126±3***
Group 2n (L-NAME)	66±1[###]	68±3	68±2	69±1
Group 3n (ODQ)	98±5	98±5	100±3	99±5
Group 4n (L-Arg)	103±3	123±4	131±3	135±3
	HYPERTENSIVE			
	Phase A	*Phase C*		
	([a])	*NaHS 1 μM*	*NaHS 10 μM*	*NaHS 100 μM*
Group 1h (vehicle)	97±1	114±7*	122±7**	124±7**
Group 2h (L-NAME)	84±6[##]	87±2	88±8	110±4**
Group 3h (ODQ)	92±8	103±9	118±10	124±10*

Table 3 - Changes in CF (% of basal value) recorded during the perfusion of hearts isolated from NTRs (upper) and SHRs (lower) with AngII (phase B), followed by cumulative concentrations of NaHS (phase C, 1-100µM), in the experimental groups 5-7.

([a]) The pharmacological treatments with enzyme inhibitors (or corresponding vehicle) added in phase A are indicated in parentheses.

Statistically different from the corresponding value recorded in Phase A (= P<0.05; ** = P<0.01; *** = P<0.001).

[§]Statistically different from the corresponding value recorded in Phase B ([§] = P<0.05; [§§] = P<0.01; [§§§] = P<0.001).

[#] Statistically different from the corresponding basal value recorded in vehicle-treated hearts ([#] = P<0.05; [###] = P<0.001).

	NORMOTENSIVE				
	Phase A	*Phase B*	*Phase C*		
	([a])	*AngII*	*NaHS 1 µM*	*NaHS 10 µM*	*NaHS 100 µM*
Group 5n (vehicle)	100±4	66±6***	78±6	98±6[§§]	131±5[§§§]
Group 6n (L-NAME)	62±1[###]	44±4***	49±3	48±2	45±3
Group 7n (ODQ)	90±13	56±7*	61±7	62±10	67±9
	HYPERTENSIVE				
	Phase A	*Phase B*	*Phase C*		
	([a])	*AngII*	*NaHS 1 µM*	*NaHS 10 µM*	*NaHS 100 µM*
Group 5h (vehicle)	98±1	66±4***	70±7	71±8	83±6[§]
Group 6h (L-NAME)	92±6	65±3**	67±4	64±4	77±6[§]
Group 7h (ODQ)	87±3[#]	76±5	82±5	79±7	95±9[§]

Table 4 - Changes in CF (% of basal value) recorded during the perfusion of hearts isolated from NTRs (upper) and SHRs (lower) with cumulative concentrations of SNP (phase C, 1nM-10µM), in the experimental groups 8n-10n, and 8h, 9h.
([a]) The pharmacological treatments with the enzyme inhibitor (or corresponding vehicle) added in phase A are indicated in parentheses.
*Statistically different from the corresponding value recorded in Phase A (** = P<0.01; *** = P<0.001).

	NORMOTENSIVE					
	Phase A	*Phase C*				
	([a])	*SNP 1nM*	*SNP 10nM*	*SNP 100nM*	*SNP 1µM*	*SNP 10µM*
Group 8n (vehicle)	101±2	111±3	116±8	125±4***	134±4***	137±5***
Group 9n (PAG)	111±7	115±5	109±7	107±8	107±5	101±3
Group 10n (L-Cys)	103±2	109±3	119±4	131±4	141±5	168±5
	HYPERTENSIVE					
	Phase A	*Phase C*				
	([a])	SNP 1nM	SNP 10nM	SNP 100nM	SNP 1µM	SNP 10µM
Group 8h (vehicle)	101±4	97±4	107±4	130±2***	139±4***	133±6***
Group 9h (PAG)	92±2	100±4	94±1	100±1**	117±3***	121±4***

Table 5 - Changes in CF (% of basal value) recorded during the perfusion of hearts isolated from NTRs (upper) and SHRs (lower) with AngII (phase B), followed by cumulative concentrations of SNP (phase C, 1nM-10μM), in the experimental groups 11-12.
([a]) The pharmacological treatments with enzyme inhibitors (or corresponding vehicle) added in phase A are indicated in parentheses.
*Statistically different from the corresponding value recorded in Phase A (** = P<0.01; *** = P<0.001).
[§]Statistically different from the corresponding value recorded in Phase B ([§] = P<0.05; [§§] = P<0.01; [§§§] = P<0.001).

	NORMOTENSIVE						
	Phase A	*Phase B*	*Phase C*				
	([a])	*AngII*	*SNP 1nM*	*SNP 10nM*	*SNP 100nM*	*SNP 1 μM*	*SNP 10μM*
Group 11n (vehicle)	101±1	69±11**	71±5	81±4	126±7[§§§]	149±6[§§§]	166±18[§§§]
Group 12n (PAG)	103±4	79±1***	84±1[§§]	86±1[§§]	99±12	108±17	112±19
	HYPERTENSIVE						
	Phase A	*Phase B*	*Phase C*				
	([a])	*AngII*	*SNP 1nM*	*SNP 10nM*	*SNP 100nM*	*SNP 1μM*	*SNP 10μM*
Group 11h (vehicle)	100±1	77±5***	80±11	80±11	84±9	104±4[§§§]	125±5[§§§]
Group 12h (PAG)	99±4	64±7***	70±12	69±12	71±11	88±10[§]	99±12[§]

Table 6 - Changes in CF (% of basal value) recorded during the perfusion of hearts isolated from NTRs with cumulative concentrations of BK (phase C, 10nM-1μM), in the experimental groups 13n-15n.

([a]) The pharmacological treatments with enzyme inhibitors (or corresponding vehicle) added in phase A are indicated in parentheses.

*Statistically different from the corresponding value recorded in Phase A (*** = P<0.001).

[#] Statistically different from the corresponding basal value recorded in vehicle-treated hearts ([###] = P<0.001).

	NORMOTENSIVE			
	Phase A	*Phase C*		
	([a])	*BK 10nM*	*BK 100 nM*	*BK 1 μM*
Group 13n (vehicle)	100±3	126±4***	158±14***	173±4***
Group 14n (L-NAME)	72±9[###]	74±4	70±5	73±3
Group 15n (PAG)	101±1	116±10	118±9	115±9

Table 7 - Changes in CF (% of basal value) recorded during the perfusion of hearts isolated from NTRs with cumulative concentrations of Nife (phase C, 1-100nM), in the experimental groups 16n-18n.

([a]) The pharmacological treatments with enzyme inhibitors (or corresponding vehicle) added in phase A are indicated in parentheses.

*Statistically different from the corresponding value recorded in Phase A (** = P<0.01; *** = P<0.001).

[#] Statistically different from the corresponding basal value recorded in vehicle-treated hearts ([###] = P<0.001).

	NORMOTENSIVE			
	Phase A	*Phase C*		
	([a])	*Nife 1nM*	*Nife 10 nM*	*Nife 100 nM*
Group 16n (vehicle)	101±1	127±3***	151±12***	147±8***
Group 17n (L-NAME)	60±2[###]	80±5**	94±8***	115±7***
Group 18n (PAG)	95±4	101±2	136±10***	147±11***

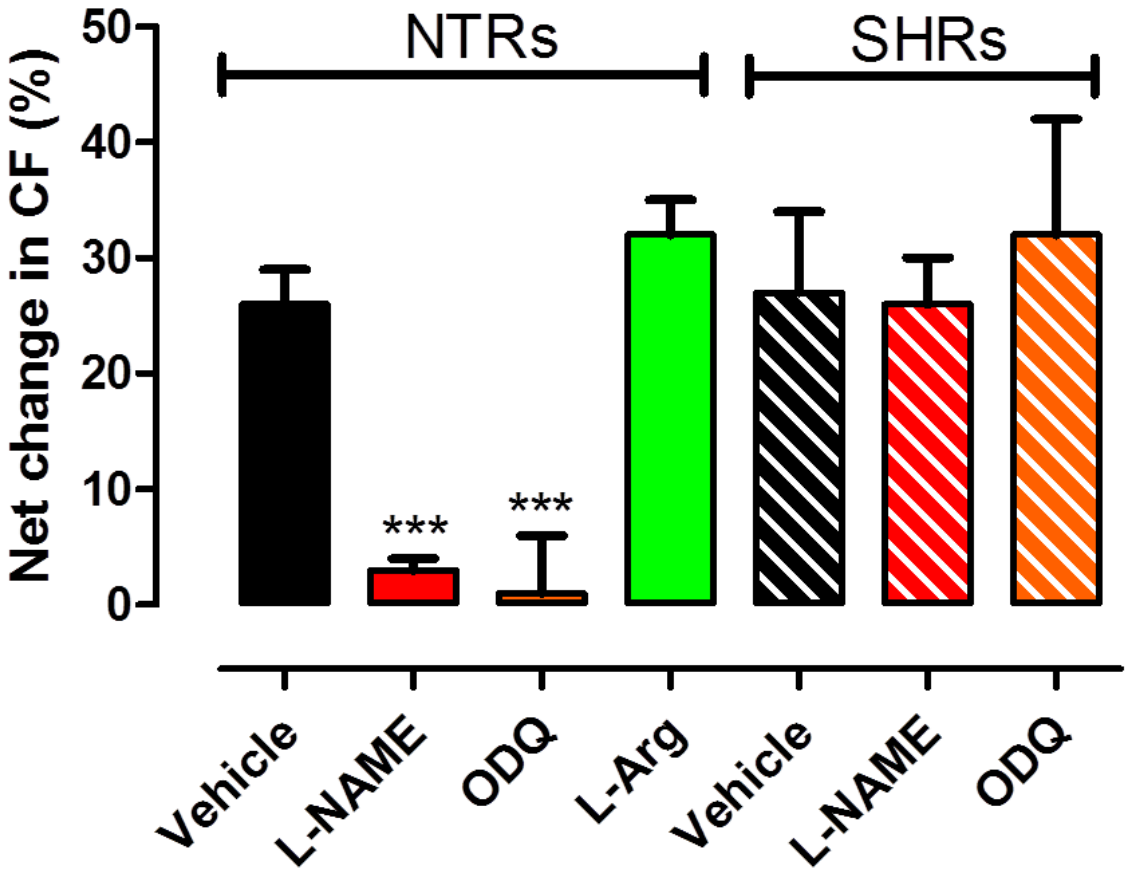
NTRs
SHRs
Net change in CF (%)
50
40
30
20
10
0
***
***
Vehicle
L-NAME
ODQ
L-Arg
Vehicle
L-NAME
ODQ

Fig. 1

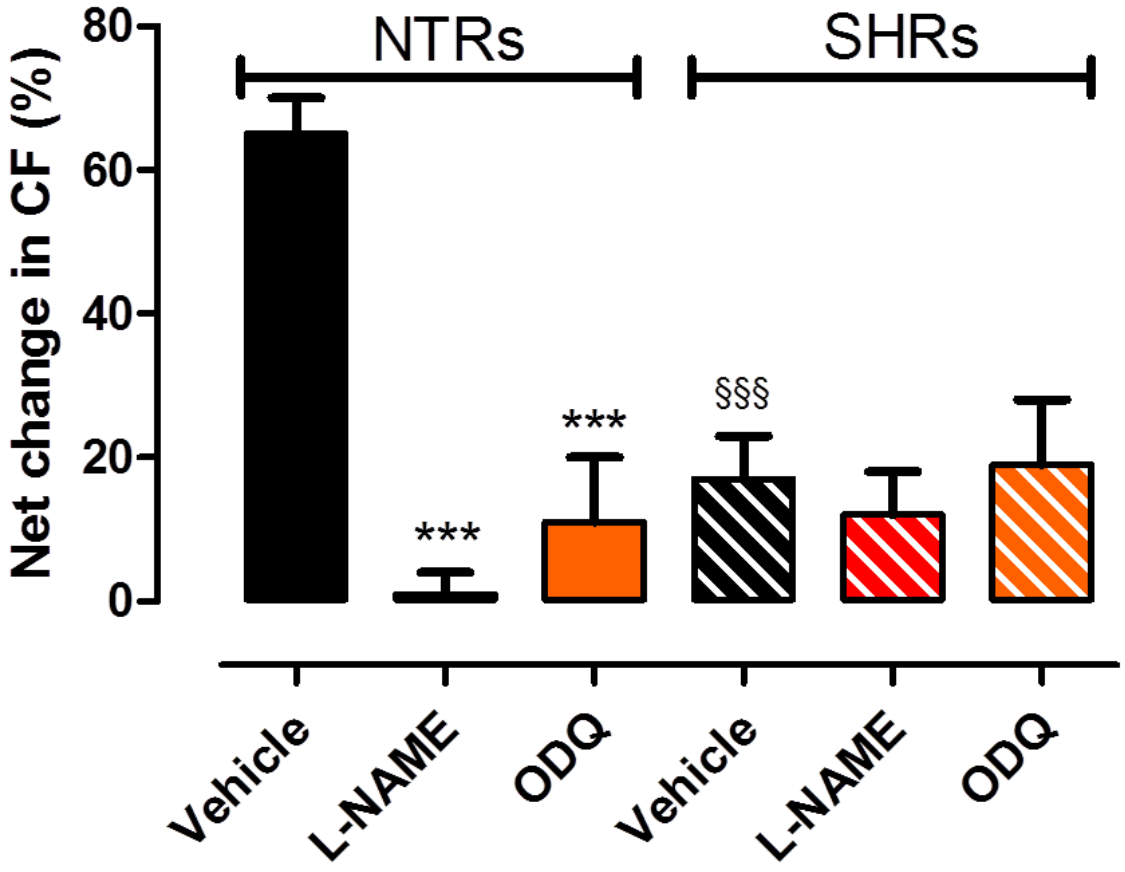
Net change in CF (%)
80
60
40
20
0
NTRs
SHRs
***
***
§§§
Vehicle
L-NAME
ODQ
Vehicle
L-NAME
ODQ

Fig. 2

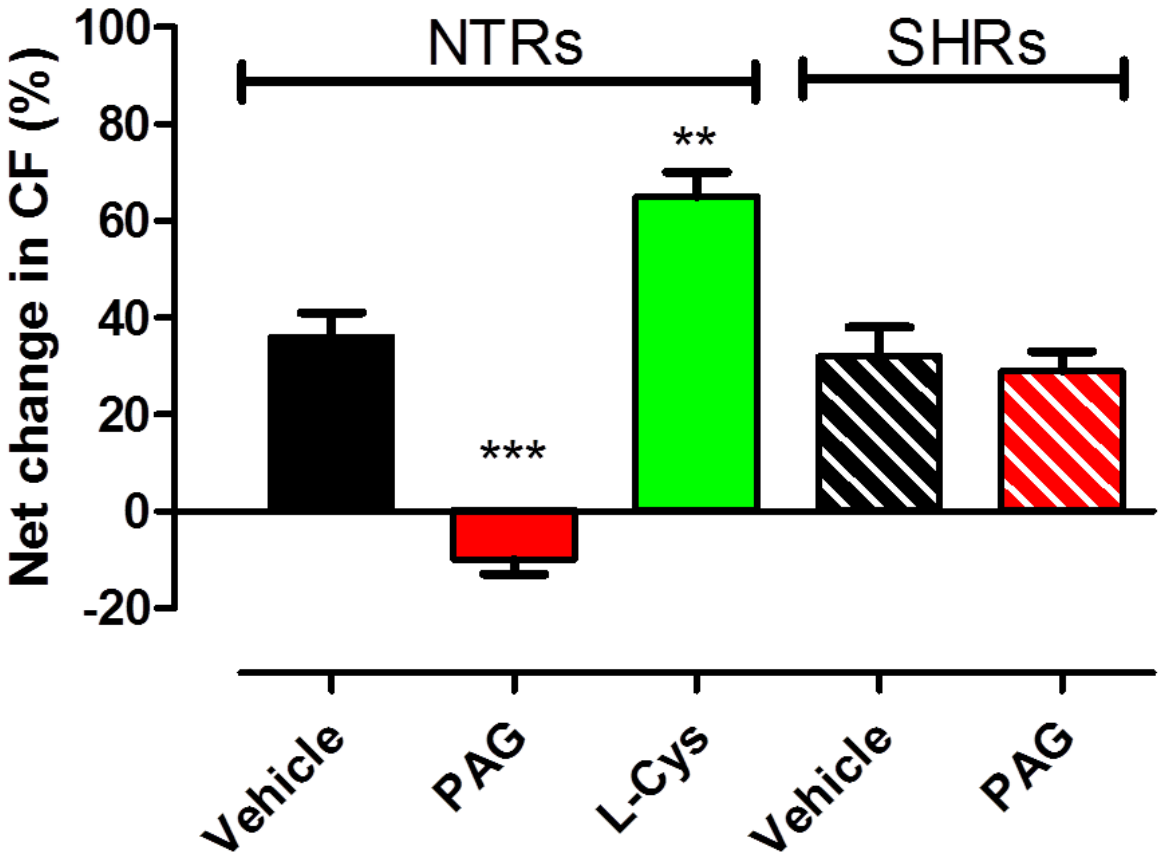
NTRs
SHRs
Net change in CF (%)
100
80
60
40
20
0
-20
**
***
Vehicle
PAG
L-Cys
Vehicle
PAG

Fig. 3

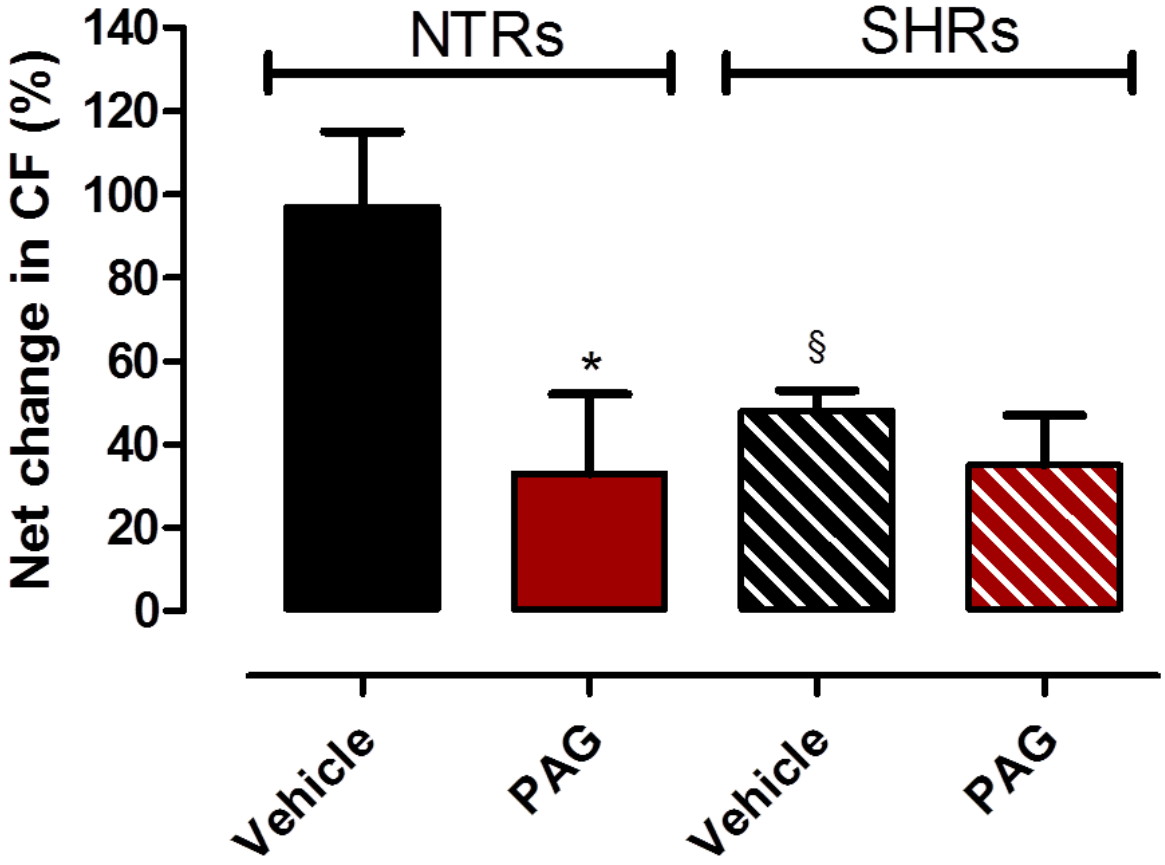
NTRs
SHRs
Net change in CF (%)
140
120
100
80
60
40
20
0
*
§
Vehicle
PAG
Vehicle
PAG

Fig. 4

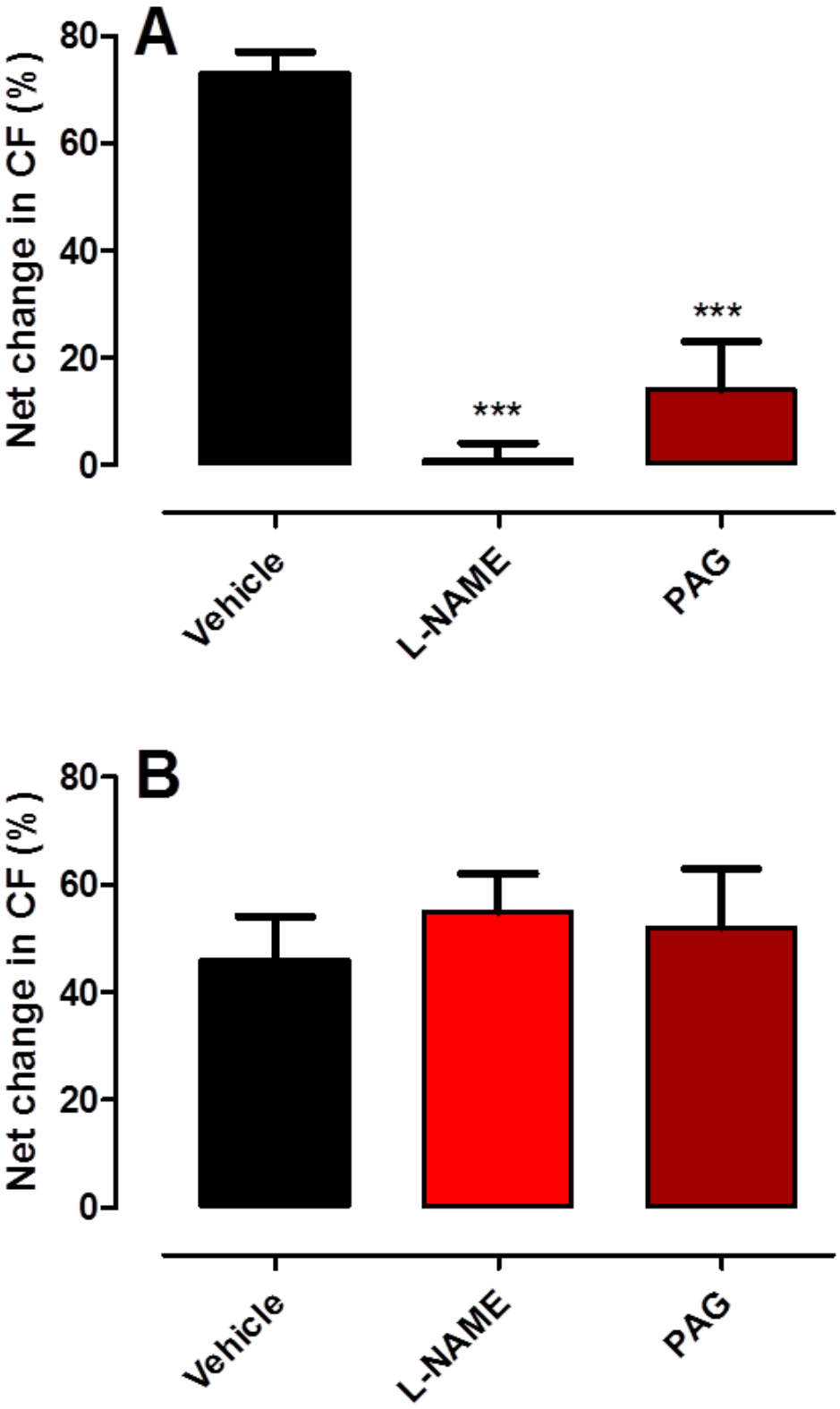
A
Net change in CF (%)
80
60
40
20
0
***
***
Vehicle
L-NAME
PAG
B
Net change in CF (%)
80
60
40
20
0
Vehicle
L-NAME
PAG

Fig. 5

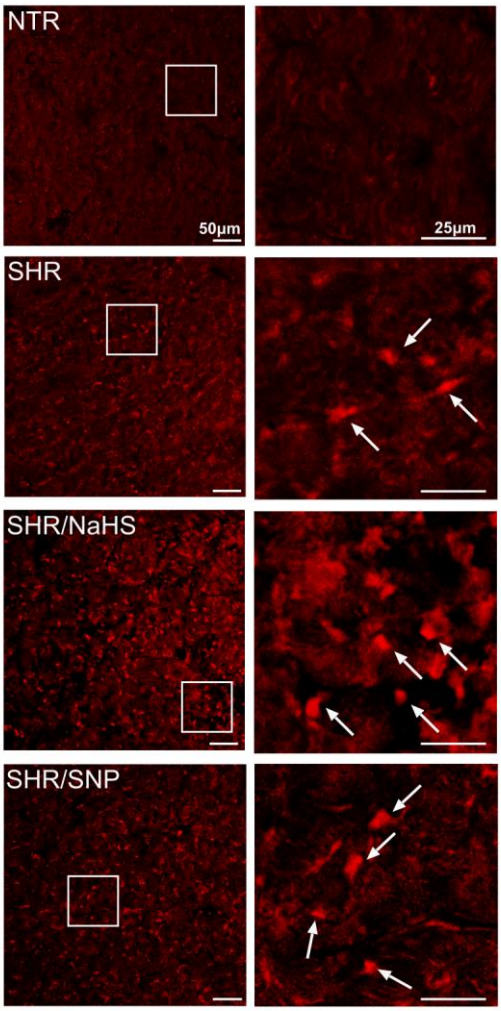

Fig. 6