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Lipid-induced glucose intolerance is driven by impaired glucose kinetics and insulin metabolism in healthy individuals

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ABSTRACT

Aims: Hypertriglyceridemia is associated with an increased risk of type 2 diabetes. We aimed to comprehensively examine the effects of hypertriglyceridemia on major glucose homeostatic mechanisms involved in diabetes progression.

Methods: In this randomized, cross-over, single-blinded study, two dual-labeled, 3-hour oral glucose tolerance tests were performed during 5-hour intravenous infusions of either 20 % Intralipid or saline in 12 healthy subjects (age 27.9 ± 2.6 years, 11 men, BMI 22.6 ± 1.4 kg/m^2) to evaluate lipid-induced changes in insulin metabolism and glucose kinetics. Insulin sensitivity, β cell secretory function, and insulin clearance were assessed by modeling glucose, insulin and C-peptide data. Intestinal glucose absorption, endogenous glucose production, and glucose clearance were assessed from glucose tracers. The effect of triglycerides on β-cell secretory function was examined in perifusion experiments in murine pseudoislets and human pancreatic islets.

Results: Mild acute hypertriglyceridemia impaired oral glucose tolerance (mean glucose: +0.9 [0.3, 1.5] mmol/L, $p = 0.008$) and whole-body insulin sensitivity (Matsuda index: −1.67 [−0.50, −2.84], $p = 0.009$). Post-glucose hyperinsulinemia (mean insulin: +99 [17, 182] pmol/L, p = 0.009) resulted from reduced insulin clearance (−0.16 [−0.32, −0.01] L min^{-1} m^{-2}, $p = 0.04$) and enhanced hyperglycemia-induced total insulin secretion (+11.9 [1.1, 22.8] nmol/m^2, $p = 0.02$), which occurred despite a decline in model-derived β cell glucose sensitivity (−41
[−74, −7] pmol min^{-1} m^{-2} mmol^{-1} L, $p = 0.04$). The analysis of tracer-derived glucose metabolic fluxes during lipid infusion revealed lower glucose clearance (−96 [−152, −41] mL/kg$_{FFM}$, $p = 0.005$), increased 2-hour oral glucose absorption (+380 [42, 718] μmol/kg$_{FFM}$, $p = 0.04$) and suppressed endogenous glucose production (−448 [−573, −123] μmol/kg$_{FFM}$, p = 0.005). High-physiologic triglyceride levels increased acute basal insulin secretion in murine pseudoislets (+11 [3, 19] pg/aliquot, $p = 0.02$) and human pancreatic islets (+286 [59, 512] pg/islet, p = 0.02).

Conclusion: Our findings support a critical role for hypertriglyceridemia in the pathogenesis of type 2 diabetes in otherwise healthy individuals and dissect the glucose homeostatic mechanisms involved, encompassing insulin sensitivity, β cell function and oral glucose absorption.

Abbreviations: AUC, Area Under the Curve; β-GS, β cell Glucose Sensitivity; β-RS, β cell Rate Sensitivity; EGP, Endogenous Glucose Production; FFA, Free Fatty Acid; GCl, Glucose Clearance; HOMA, Homeostatic Model Assessment of Insulin Resistance; iAUC, incremental AUC; ISR, Insulin Secretion Rate; ISR@5, ISR at a fixed glucose concentration of 5mmol/L; OGTT, Oral Glucose Tolerance Test; RaO, Rate of appearance of Oral glucose; VLDL, Very Low Density Lipoprotein.

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1. Introduction

In the early phase of the progression to type 2 diabetes, insulin bioavailability is typically increased due to a rise in insulin secretion and a parallel decline in peripheral and hepatic insulin sensitivity [1]. The biological signals that generate and sustain insulin hypersecretion and insulin resistance in the absence of significant hyperglycemia are largely unknown. Plausible candidates are lipid substrates and particularly free fatty acids (FFA), whose detrimental effects on glucose metabolism have been demonstrated both *in vitro* and *in vivo* [2]. However, the pathogenetic role of moderately high FFA levels, such as those found in prediabetes, has been recently questioned [3]. Among other lipid species, elevated plasma triglycerides have been traditionally considered a mere consequence of obesity and insulin resistance, their negative effects being supposedly mediated by their circulating metabolites FFA. Nevertheless, large population-based studies demonstrated that high plasma triglycerides are predictive of glucose intolerance and type 2 diabetes in a dose-dependent fashion, regardless of insulin resistance and FFA [4–8]. Studies using a Mendelian randomization approach found associations between genetically determined hypertriglyceridemia and risk of diabetes [9,10]. Finally, a growing body of evidence in humans and animal models (reviewed in ref. [11]) strongly suggests that high triglyceride availability could directly contribute to early derangements of glucose homeostatic mechanisms, including insulin resistance and chronic insulin hypersecretion.

Hypertriglyceridemia produced by an acute or prolonged (12 to 48 h) *i.v.* infusion of a lipid emulsion was associated with increased plasma glucose and insulin levels and a decline in indices of insulin sensitivity in most previous studies [12–18]. Lipid-induced hyperinsulinemia may be driven by enhanced β cell function and/or by reduced insulin clearance. Mild chronic hypertriglyceridemia was associated with increased insulin secretion at fasting and after a glucose load, irrespective of potential confounders, in two large cohorts of adults [19] and youths [20]. Furthermore, an impairment in insulin clearance by lipid infusion has been reported in some [16] but not all [14] available studies, though this important homeostatic mechanism has been often overlooked. Additionally, it has been postulated that triglyceride availability may influence post-prandial glucose kinetics also by increasing the rate of systemic appearance of oral glucose and by inhibiting the physiological suppression of endogenous glucose production [13]. Given the proposed multiple actions of triglycerides on relevant aspects of whole-body glucose metabolism, it is challenging to dissect the specific effect of triglycerides on each of the main determinants of glucose homeostasis, all of which are entangled in a complex interplay. To achieve this result, an integrated physiology approach and the use of sophisticated mathematical models of β cell function and glucose kinetics are required, which were not implemented in previous human studies.

Based on the available evidence, we hypothesized that plasma triglycerides are active determinants of glucose tolerance and directly affect multiple major glucose homeostatic mechanisms involved in diabetes progression. Hence, in normally-glucose tolerant subjects, we quantified the effects of mild acute hypertriglyceridemia induced by an *i.v.* lipid emulsion infusion on glucose tolerance, model-derived β cell function, endogenous insulin clearance, whole-body and hepatic insulin sensitivity, and tracer-derived glucose metabolic fluxes during a dual-labeled oral glucose tolerance test (OGTT). Thereafter, we directly examined the acute effect of high physiological triglyceride levels on basal and glucose-stimulated insulin secretion in murine β cells and human pancreatic islets *in vitro*. A better understanding of the complex interrelationship between hypertriglyceridemia, insulin metabolism and glucose kinetics can provide new insights into the mechanisms that contribute to the pathogenesis of type 2 diabetes in otherwise healthy individuals.

2. Methods

2.1. Clinical study participants

We conducted a cross-over, randomized clinical study in fourteen volunteers recruited among students and fellows attending the clinic of the Unit of Clinical Nutrition and Dietetics at the University of Pisa (Italy). The inclusion criteria were age 18–65 years, BMI 18–25 kg/m^2, both women and men. At screening, information regarding medical history and drug use was collected using standardized self-reported questionnaires. Fasting blood samples were collected for routine blood tests and glycated hemoglobin (HbA_{1c}) measurements. Brachial blood pressure was measured three times in subjects seated for at least 10 min, and the last two measurements were averaged for analysis. Individuals with chronic or acute diseases, taking medications influencing glucose or lipid metabolism, and pregnant women were excluded. Subjects were classified as having normal glucose tolerance (NGT) according to the current diagnostic criteria [21]. Among 14 volunteers initially screened, 2 subjects had impaired glucose tolerance at the control OGTT and were excluded from the analyses.

The study was approved by the local Human Ethics Committee (protocol n. 13053_NATALI) and conducted in accordance with the principles expressed in the Declaration of Helsinki. All subjects provided written informed consent before enrollment.

2.2. Metabolic tests

Each participant underwent a total of two OGTTs, modified with the infusion of lipid/saline and the use of two stable glucose isotopes, during two separate visits 2–4 weeks apart (Fig. 1A). Prior to the OGTT, all participants were prescribed a weight-maintenance diet consisting of at least 250 g of carbohydrate per day for 3 days and a standardized pre-test evening meal. Furthermore, they were asked to keep their physical activity level as similar as possible between the two study visits, avoiding strenuous physical activity for 3 days prior to the OGTT. At 08:00 am after an overnight fast (12*h*), participants were admitted to our Clinical Research Unit. A 20-gauge polyethylene cannula was inserted into an antecubital vein for the infusion of all test substances. A second cannula was inserted retrogradely into an ipsilateral wrist vein for blood sampling, and the hand was kept wrapped in a heated blanket to achieve the arterialization of venous blood. At time −120 min, volunteers were randomized by a computer generated random number list to receive a 5 h primed (375 mL/m^2)-continuous (25 mL h^{-1} m^{-2}) *i.v.* infusion of 20 % fat emulsion (Intralipid 20 %, Baxter, Deerfield, IL) or normal saline (0.9 % sodium chloride), together with a 5 h primed (28 μmol/kg)-continuous (0.28 μmol min^{-1} kg^{-1}) infusion of 6,6-[$^2H_2$]glucose (Cambridge Isotope Laboratories, Tewksbury, MA). Participants were blinded as to the order of the two infusions (Intralipid *vs* saline). After 2 h (time 0 min), participants consumed within 5 min an oral glucose drink consisting of 147 mL of 50 % dextrose solution (wt/vol) enriched with 1.5 g of [U-^{13}C] glucose (Cambridge Isotope Laboratories). The oral glucose tracer was unintentionally omitted from the glucose drink of the control OGTT in one subject. Arterialized blood samples were collected throughout the test at time −120, −90, −60, −30, −15, 0, 15, 30, 45, 60, 90, 120, 150, 180 min to measure plasma glucose, insulin, C-peptide, glucagon, free fatty acids (FFA), triglycerides, and glucose tracer enrichments.

2.3. Glucose kinetics

Glucose clearance (GCl), endogenous glucose production (EGP) and the rate of oral glucose appearance (RaO) were assessed from the time course of the plasma tracer/tracee ratio of 6,6-[$^2H_2$]glucose and [U-^{13}C] glucose using a previously described model [22]. EGP mostly reflects hepatic glucose output and RaO depends on the rates of intestinal glucose absorption and splanchnic glucose uptake.

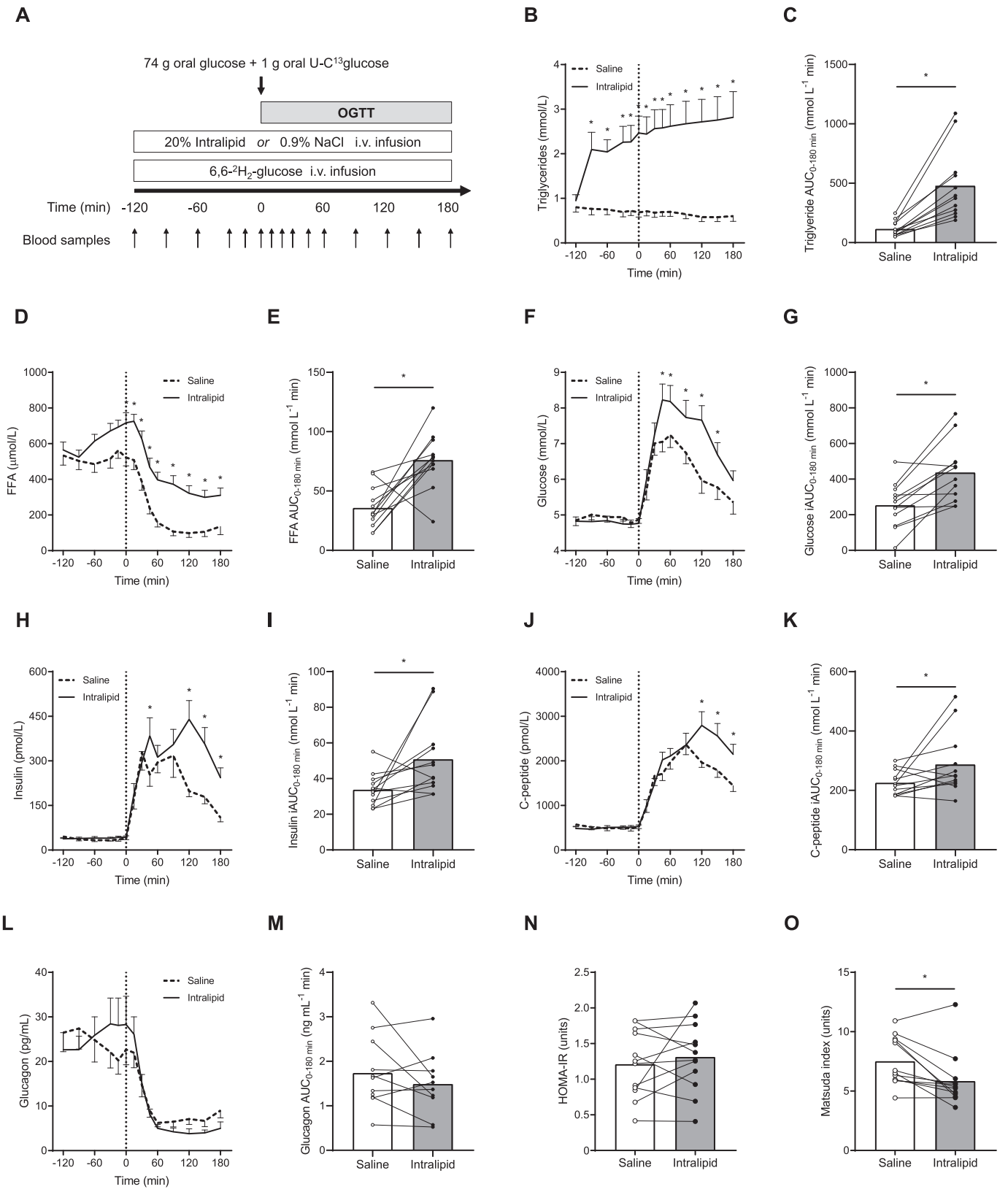

Fig. 1. Plasma metabolites, hormones, and insulin sensitivity in response to lipid infusion and glucose ingestion. The clinical study consisted in repeated dual-labeled, 3-hour oral glucose tolerance tests (OGTTs, from time 0 to 180 min) during 5-hour *i.v.* infusions of either saline or Intralipid (A). Plasma concentrations and 3-hour post-glucose AUCs of triglycerides (B-C), FFA (D-E), glucose (F-G), insulin (H-I), C-peptide (J-K), glucagon (L-M), and insulin sensitivity indices at fasting (N) and during the OGTT (O) in 12 volunteers with normal glucose tolerance. In B, D, F, H, J, and L, values represent the mean ± SEM; *p* values were determined by two-way ANOVA followed by multiple pairwise comparisons using Tukey's *post hoc* tests when appropriate. In C, E, G, I, K, M, N, and O, individual values and group means are shown; *p* values were determined by Wilcoxon signed-rank tests. * $P < 0.05$.

2.4. Beta cell function modeling

Insulin secretion rate (ISR) was estimated by C-peptide deconvolution [23]. Parameters of β cell function were calculated by mathematical modeling of ISR and glucose concentrations during the OGTT, as previously reported [24,25]. The relationship between glucose and ISR is described as the sum of two components. The first component represents the dependence of ISR on absolute glucose concentration at any time point. The quasi-linear dose–response function relating the two variables is described by a slope, named β cell glucose sensitivity (β-GS), and by ISR at a fixed glucose concentration of 5 mmol/L (ISR@5). The static dose-response function can be modulated over time by several factors (*i.e.* persistent hyperglycemia, non-glucose substrates, gastrointestinal hormones, and neurotransmitters), which are collectively modeled as a potentiation multiplying factor. The potentiation factor is set to be a positive function of time and to average the value 1 during the OGTT. The ratios of the values at 160–180 min and 100–120 min *vs* 0–20 min are named potentiation factors and used to express this potentiation effect. The second insulin secretion component represents the dynamic dependence of ISR on the rate of change of glucose concentration and is named β cell rate sensitivity (β-RS).

2.5. Insulin clearance

Endogenous insulin clearance, which largely reflects hepatic insulin clearance, was calculated as the ratio between fasting ISR and plasma insulin level (ISR_{fast}/Ins_{fast}) and as the ratio of their areas under the curve (AUC) during the OGTT (ISR_{AUC}/ Ins_{AUC}), as previously reported [26].

2.6. Insulin sensitivity

Fasting insulin sensitivity was estimated using the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) index, calculated as (fasting glucose × fasting insulin /22.5). Insulin sensitivity during the OGTT was estimated using the Matsuda index, calculated as 10,000/([fasting glucose × fasting insulin] × [mean glucose × mean insulin during OGTT])$^{1/2}$. Additionally, we calculated indices of peripheral insulin sensitivity and hepatic insulin resistance using glucose kinetics data, as previously reported [27,28]. Peripheral insulin sensitivity was estimated as the GCl normalized to plasma insulin levels during the OGTT (GCl/Ins), calculated as the ratio between GCl_{AUC} and Ins_{AUC}. Hepatic insulin resistance at fasting was estimated as the product of EGP and plasma insulin at time 0 min (EGP × FPI), as proposed by DeFronzo [28]. Hepatic insulin resistance during the OGTT was estimated as the product of EGP_{AUC} and ISR_{AUC} (EGP × ISR), the latter being a marker of portal insulin levels during the OGTT [27].

2.7. In vitro studies

The Murine INsulinoma (MIN6) β cell line was a kind gift from Dr. Y. Oka and Professor J.I. Miyazaki (University of Tokyo, Tokyo, Japan). Human islets were isolated from a 47-year-old male donor with a BMI of 21 kg/m^2 at the King's College Hospital (London, UK). Detailed methods of MIN6 β cell culture, isolation and culture of human pancreatic islets, assessment of MIN6 apoptosis by measurement of caspase 3/7 activity, and perifusion experiments using MIN6 pseudoislets and human pancreatic islets are reported in Supplemental Materials.

2.8. Analytical procedures

In the clinical study, plasma glucose was measured immediately by the glucose-oxidase technique (Beckman Glucose Analyzer II, Beckman Instruments, Fullerton, CA). Insulin and C-peptide measurements were performed by electrochemiluminescence (COBAS e411, Roche, Indianapolis, IN). Plasma glucagon was assessed using a Multiplex technique (Biorad Laboratories, Hercules, CA). 6,6-[$^2H_2$]glucose and [U-^{13}C] glucose were measured by gas chromatography–mass spectrometry (GC–MS). Plasma FFA and triglycerides were assayed by standard spectrophotometric methods on a Synchron Clinical System CX4 (Beckman Instruments). An in-house radioimmunoassay was used for measuring the amount of insulin secreted into the media during perifusion experiments [29].

2.9. Statistical analysis

Continuous variables were tested for normality using the Shapiro–Wilk test. Data are reported as mean ± SD or median [interquartile range] if not normally distributed, unless otherwise stated. Differences between the lipid and saline infusion were analyzed using paired Student's *t*-tests or Wilcoxon signed-rank tests, as appropriate, and reported as mean difference [95 % confidence interval]. Group differences in experiments *in vitro* were analyzed using Kruskal-Wallis test followed by Dunn's *post hoc* pairwise comparisons. Repeated measures were analyzed by two-way ANOVA including study condition (lipid *vs* saline infusion or lipoprotein *vs* control), time, and study × time interaction effects as factors. If this test revealed statistically significant effects, multiple pairwise comparisons were performed using Tukey's post-hoc tests. AUC and incremental AUC (iAUC) were calculated using the trapezoidal rule. Analyses were performed using JMP Pro 13.2.1 (SAS, Cary, NC) at a two-sided α level of 0.05.

3. Results

3.1. Clinical study participants

Twelve volunteers completed the clinical study (mean age 27.9 ± 2.6 years [range 22.9–31.4 years], 11 men and 1 woman, BMI 22.6 ± 1.4 kg/m^2 [range 18.5–23.8 kg/m^2]). They were normoglycemic (fasting plasma glucose 4.8 ± 0.4 mmol/L, HbA1c 31 ± 2 mmol/mol), normotensive (systolic blood pressure 114 ± 13 mmHg, diastolic blood pressure 77 ± 6 mmHg), and had a normal plasma lipid profile (total cholesterol 4.2 ± 0.6 mmol/L, HDL cholesterol 1.5 ± 0.3 mmol/L, LDL cholesterol 2.5 ± 0.5, triglycerides 0.9 [0.7–1.2] mmol/L). None of them was taking any medications or dietary supplements. The lipid infusion was well tolerated without any adverse effect in all participants.

3.2. Mild acute hypertriglyceridemia impairs glucose tolerance and insulin sensitivity

Fasting pre-infusion plasma triglycerides, FFA, glucose, insulin, C-peptide and glucagon were not different between the two study visits ($p > 0.51$ for all; Fig. 1B-M). The lipid infusion markedly increased plasma triglyceride concentration (time 0 min: +1.8 [1.1, 2.4] mmol/L, $p = 0.0005$) towards high-physiological levels (approximately 2.5 mmol/L; Fig. 1B) and induced a progressive small increase in plasma FFA (time 0 min: +194 [23, 365] μmol/L, $p = 0.03$; Fig. 1D), without affecting plasma glucose (Fig. 1F), insulin (Fig. 1H), C-peptide (Fig. 1J), and glucagon levels (Fig. 1L; $p > 0.37$ for all). During the OGTT (time 0 to 180 min), triglyceride concentrations remained stable and approximately four times higher during lipid infusion compared with saline (AUC: +363 [206, 520] mmol L^{-}1 min, $p = 0.0003$; Fig. 1B-C), while FFA were suppressed to a similar extent during both study conditions (time × study effect: $p = 0.61$; AUC: +40 [18, 62] mmol L1 min, $p = 0.002$; Fig. 1D-E). The lipid infusion was associated with markedly increased plasma glucose (iAUC: +184 [93, 274] mmol L1 min, $p = 0.001$; Fig. 1F-G), insulin (iAUC: +17.0 [2.2, 31.9] nmol L^{-1} min, $p = 0.03$; Fig. 1 H-I) and C-peptide excursions (iAUC: +61.6 [2.8, 126.0] nmol L^{-1} min, p = 0.03; Fig. 1 J-K) during the OGTT, whereas the glucagon response was not different between the two study conditions ($p = 0.32$; Fig. 1L-M). Whole-body insulin sensitivity was not affected by lipid infusion at baseline (HOMA-IR: $p = 0.57$; Fig. 1N) but was

markedly impaired during the OGTT, as indicated by the Matsuda index (−1.67 [−0.50, −2.84], $p = 0.009$; Fig. 1O) and by tracer-derived indices described below.

3.3. Mechanisms of lipid-induced hyperinsulinemia: β cell function and insulin clearance

Paralleling the glucose and C-peptide curves, the ISR was not affected by lipid infusion before glucose ingestion (time 0 min: $p = 0.85$; Fig. 2A-B), whereas it was significantly enhanced during the OGTT in the lipid-infusion visit (AUC: +11.9 [1.1, 22.8] nmol/m^2, $p = 0.02$; Fig. 2C). Despite the relative insulin hypersecretion during lipid infusion, the lower slope of the glucose-ISR dose-response curve (Fig. 2D), *i.e.,* the reduction in β-GS (−41 [−74, −7] pmol min^{-1} m^{-2} mmol^{-1} L, $p = 0.04$; Fig. 2E), indicated a significant decline in glucose-dependent insulin secretion. Among other indices of β cell function, the potentiation ratio was increased during lipid infusion in the later phase of the OGTT (180 min/baseline ratio: +0.6 [0.1, 1.0], p = 0.02; Fig. 2G-I), while ISR@5 ($p = 0.47$; Fig. 2F) and β-RS ($p = 0.46$; Fig. 2J) were not affected.

Compared with saline, lipids reduced insulin clearance both at baseline (time 0 min: −0.29 [−0.44, −0.14] L min^{-1} m^{-2}, $p = 0.0005$) (Fig. 2K) and after glucose ingestion (−0.16 [−0.32,-0.01] L min^{-1} m^{-2}, $p = 0.04$) (Fig. 2L).

3.4. Effects of hypertriglyceridemia on glucose kinetics

Whole-body glucose clearance (GCl) and endogenous glucose production (EGP) were not affected by lipids before glucose ingestion (time 0 min: $p = 0.34$ and $p = 0.11$, respectively; Fig. 3). Conversely, all glucose metabolic fluxes were influenced by lipids during the OGTT. GCl was consistently reduced throughout the OGTT (AUC: −96 [−152, −41] mL/kg$_{FFM}$, $p = 0.005$; Fig. 3A-B), even after correction for plasma insulin levels (GCl/Ins: AUC: −5.5 [−8.2, −2.8] mL kg$^{-1}_{FFM}$ nmol^{-1} L, $p = 0.002$; Fig. 3C), being GCl/Ins an index of whole-body insulin sensitivity [27]. EGP was significantly more suppressed by lipids (AUC: −448 [−573, −123] μmol/kg$_{FFM}$, p = 0.005; Fig. 3D-E), possibly as a consequence of hyperinsulinemia (Supplemental Fig. 1). There were no differences in hepatic insulin resistance at baseline (EGP × FPI: $p = 0.75$) and during the OGTT (EGP × ISR: $p = 0.58$; Fig. 3F). Furthermore, the rate of oral glucose appearance (RaO) was greater over the first 120 min of the OGTT during lipid infusion (AUC: +380 [42, 718] μmol/kg$_{FFM}$, p = 0.04; Fig. 3G-I).

3.5. High physiological triglyceride levels enhance dynamic insulin secretion in human pancreatic islets

Preliminary experiments in mouse clonal MIN6 β cells demonstrated that high physiological triglyceride concentrations (2.5 mmol/L) did not stimulate basal or cytokine-induced apoptosis, measured by caspase 3/7 activity, after short (24-h) or prolonged (72-h) exposure to triglyceride-rich very low density lipoproteins (VLDL), while lipotoxicity was induced by supraphysiological triglyceride concentrations (Fig. 4A-B). We next examined whether acute exposure to human VLDL has functional consequences in insulin secreting cells (Fig. 4C-H). In perifusion studies, 2.5 mmol/L VLDL caused a rapid and maintained increase in insulin secretion from MIN6 pseudo-islets (Fig. 4C) and human islets (Fig. 4F) at a basal glucose concentration, which returned to baseline shortly after VLDL infusion was suspended. The total ISR during VLDL infusion at basal glucose concentration (time 10 to 30 min) was 33 % (+11 [3,19] pg/aliquot, $p = 0.02$; Fig. 4D) and 170 % (+286 [59, 512] pg/islet, p = 0.02; Fig. 4G) greater in MIN6 pseudo-islets and human islets, respectively, compared with non-exposed control groups. In human islets, VLDL-induced ISR was also enhanced at stimulatory glucose concentration, particularly in the later phase of the perifusion, with a numerical increase in the total ISR at time 60–80 min (+174 [−72, 419] pg/islet, $p = 0.13$; Fig. 4H). Repeated measure analysis for the whole ISR profile showed a significant effect of the interaction between group (VLDL *vs* control) and time (MIN6 pseudo-islets: $p = 0.003$; human islets: $p < 0.0001$).

4. Discussion

The purpose of this hypothesis-driven translational research project was to provide a comprehensive evaluation of the effects of acute high triglyceride availability on glucose tolerance and insulin metabolism, with the final aim to unravel a plausible pathogenetic mechanism of diabetes progression in individuals with normal glucose tolerance.

The major findings from our clinical study can be summarized as follows (Fig. 5). In a low-risk population without glucose alterations and insulin resistance, high triglyceride levels did not affect fasting glucose but markedly impaired oral glucose tolerance. Postload hyperglycemia was driven by a significant deterioration of whole-body (but not hepatic) insulin sensitivity, leading to lower glucose disposal in spite of higher insulin levels. The relative hyperinsulinemia induced by lipids resulted from two processes. First, enhanced insulin secretion in response to the oral glucose load, which was mainly due to the higher prevailing plasma glucose levels during the glucose challenge. Second, reduced insulin clearance, allowing more of the secreted insulin to reach and remain into the systemic circulation. Two intrinsic changes in model-derived β cell secretory function were also noted, independent of insulin resistance and glucose levels: a decline in β cell glucose sensitivity and an increase in potentiation. Finally, along with the expected reduction in glucose disposal, the analysis of glucose kinetics revealed two unanticipated changes in tracer-derived glucose metabolic fluxes, namely a more suppressed glucose production (EGP) and faster intestinal absorption (RaO), which have opposite effects on plasma glucose levels. Our data demonstrate that even mild acute hypertriglyceridemia in the absence of major FFA modifications has negative effects on glucose homeostasis in healthy individuals, encompassing whole-body insulin resistance, dysfunction of static β cell response to glucose, and enhanced oral glucose appearance, whose effects are attenuated by hyperglycemia-driven insulin hypersecretion and enhanced β cell potentiation.

Previous human studies on the alterations of glucose tolerance induced by lipids have tested supraphysiological lipid concentrations and focused on the effects of FFA. For this reason, the infusion of lipid emulsions was higher compared with our study [13,14] and/or combined with that of heparin, which activates the lipoprotein lipase and promotes the metabolism of triglycerides to FFA and glycerol [30]. In normal weight young adults [13] and children [14], a lipid infusion rate higher than our study (0.015–0.020 mL kg^{-1} min^{-1}) without heparin induced a marked increase in fasting plasma triglycerides (up to 4.4 mmol/L) combined with a 3-fold increase in plasma FFA, preventing the physiological suppression of FFA below baseline levels after glucose ingestion [13]. Moreover, heparin infused in some studies is known to interact with insulin biological activity [31] and to release a great amount of glycerol from triglyceride metabolism, which is a substrate for gluconeogenesis. These factors hindered the possibility to clearly dissect the effects of triglycerides from that of FFA and other circulating substrates in the available studies. In contrast, a slower lipid infusion rate was used in our clinical experiment to increase plasma triglyceride levels towards high physiological values observed in the general population (2.5 mmol/L) [19] and to minimize the parallel increase in FFA due to triglyceride metabolism, which still naturally occurred (+42.8 [−12.1, +81.0] % from baseline values).

Our current findings demonstrate that high triglyceride levels *per se* are able to deteriorate glucose tolerance and insulin sensitivity. In line with this observation, an increase in glucose levels and markers of insulin resistance induced by acute lipid infusions without heparin has been previously reported [12,13]. More recently, two studies in youths with a 3 h [14] or 12 h [15] lipid infusion and a study in elderly obese individuals with a 24 h lipid infusion [16] further confirmed a decline in

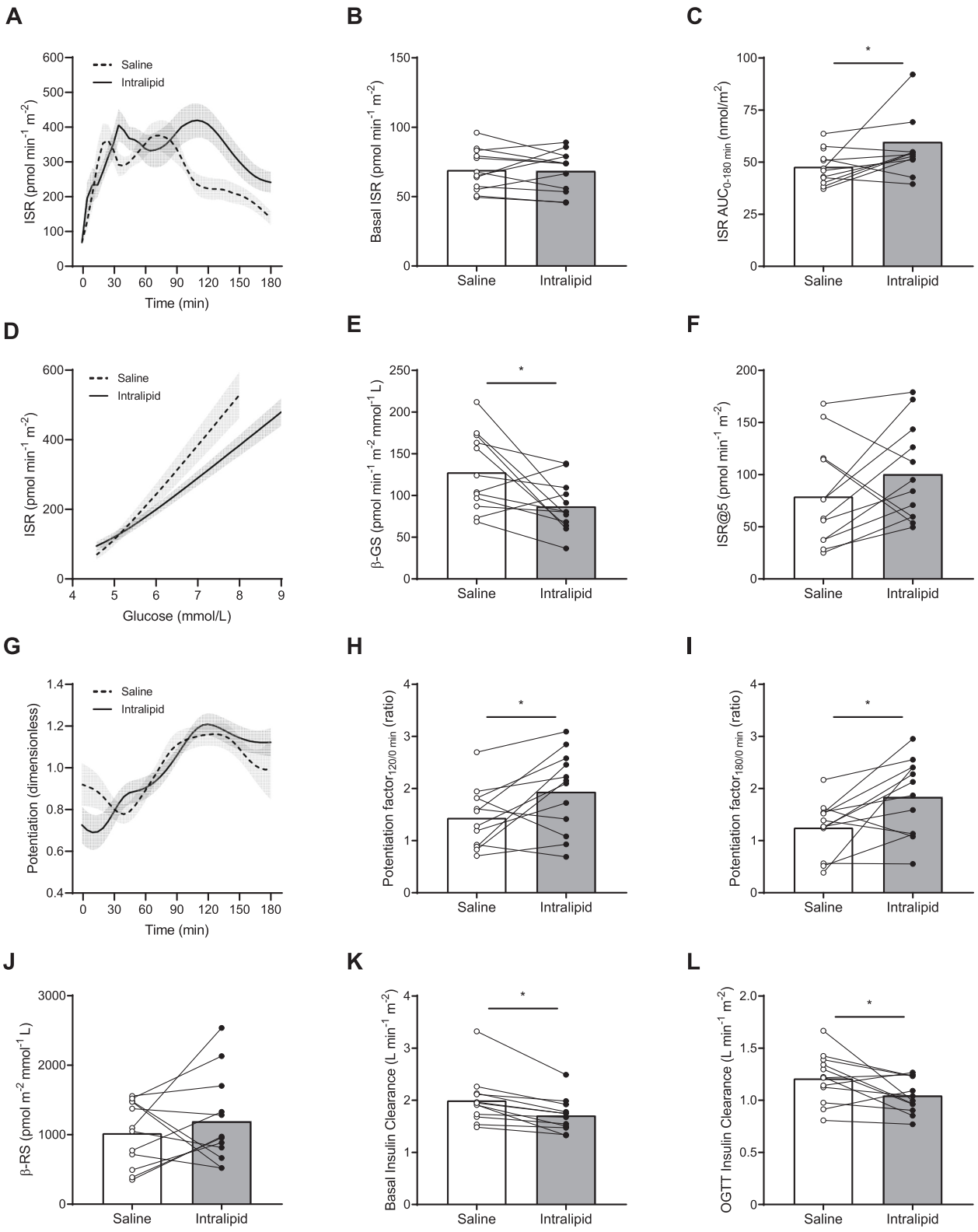

Fig. 2. Insulin secretion, β cell function, and insulin clearance in response to lipid infusion and glucose ingestion. Insulin secretion rate (ISR) estimated from C-peptide concentrations in response to 3-hour oral glucose tolerance tests (OGTTs) during *i.v.* infusions of either saline or Intralipid in 12 volunteers with normal glucose tolerance (A-C). Relationship between plasma glucose concentrations and ISR (D). Model-derived parameters of β cell function, including β cell glucose sensitivity (β-GS) (E), ISR at 5 mmol/L glucose (ISR@5) (F), potentiation (G-I), and β cell rate sensitivity (β-RS) (J). Insulin clearance at baseline (K) and during the OGTT (L). In A, D, and G, lines represent the mean and shadows represent the SEM. In B, C, E, F, H, I, J, K, and L, individual values and group means are shown; *p* values were determined by Wilcoxon signed-rank tests. * $P < 0.05$.

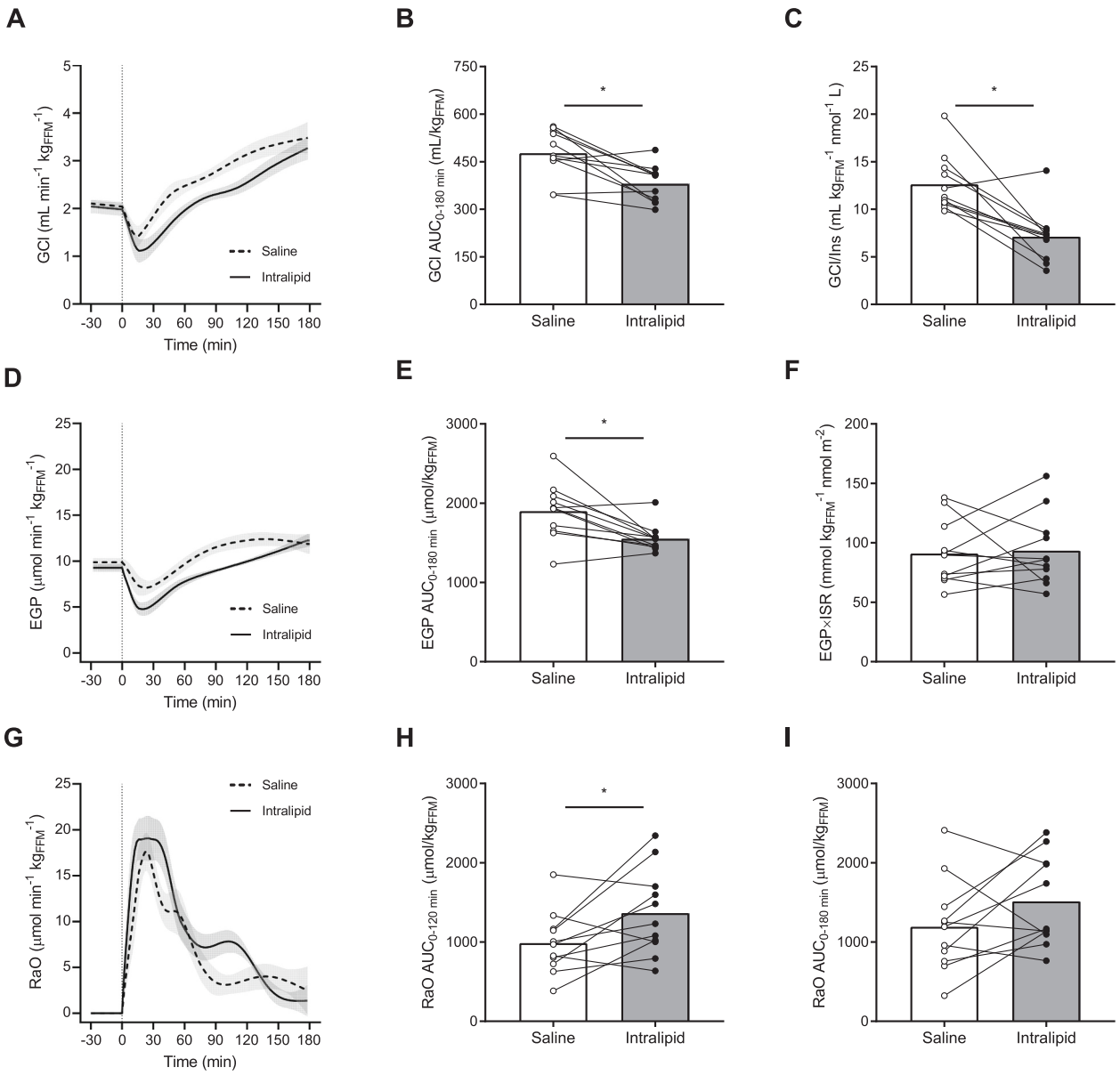

Fig. 3. Glucose kinetics in response to lipid infusion and glucose ingestion. Glucose clearance (GCl), endogenous glucose production (EGP), and rate of appearance of oral glucose (RaO) were assessed from the time course of the plasma tracer/tracee ratio of 6,6-[$^{2}H_{2}$]glucose and [U-^{13}C]glucose in response to 3-hour oral glucose tolerance tests (OGTTs) during *i.v.* infusions of either saline or Intralipid in 11 volunteers with normal glucose tolerance. Changes in GCl (A), GCl AUC during the OGTT (GCl $AUC_{0–180\ min}$) (B), GCl adjusted for plasma insulin concentrations (C), EGP (D), EGP AUC during the OGTT (EGP $AUC_{0–180\ min}$) (E), EGP adjusted for ISR as a proxy of portal insulin concentrations (F), RaO (G), and RaO AUC during the first 120 min (RaO $AUC_{0–120\ min}$) (H) and 180 min of the OGTT (RaO $AUC_{0–180\ min}$) (I). In A, D, and G, lines represent the mean and shadows represent the SEM. In B, C, E, F, H, and I, individual values and group means are shown; *p* values were determined by Wilcoxon signed-rank tests. * $P < 0.05$.

insulin sensitivity induced by lipids. In addition, a more sustained (4 days) lipid infusion caused a rise in fasting and mean 48 h plasma glucose and induced insulin resistance in healthy normal glucose tolerant individuals [17].

Along with insulin sensitivity, we hypothesized that lipid availability may influence insulin secretion under basal and high-glucose conditions. In fasting conditions, circulating triglycerides are mainly transported into the core of VLDL, which are water-soluble and can directly interact with β cells through a receptor-mediated mechanism [32–34]. Human and murine β cells, but not α cells, express functional receptors for VLDL and can internalize these lipoproteins [32,34]. A direct association between fasting triglycerides or triglyceride-rich VLDL and surrogate markers of β cell function was previously observed in two large cohorts of adults [19] and youths [20], independent of potential confounders. Additionally, higher plasma insulin levels in response to different oral or *i.v.* glucose challenges were observed in most [13–15,17] but not all previous studies [16] in non-diabetic individuals undergoing lipid infusions. In our clinical study, a prompt and sustained rise in plasma triglycerides had negligible effects on fasting ISR and plasma insulin levels, while it enhanced ISR and plasma insulin in response to the oral glucose load. The relative insulin hypersecretion during the OGTT was driven by the higher prevailing glucose levels, as the relationship between glucose concentration and ISR was impaired by triglycerides. A deterioration of β cell responsiveness to glucose is consistent with previous studies showing that the ISR adjusted for insulin resistance is reduced during lipid infusions, particularly in individuals with genetically determined diabetes risk [16,17]. The more sustained hyperglycemia during lipid infusion, in turn, may explain the

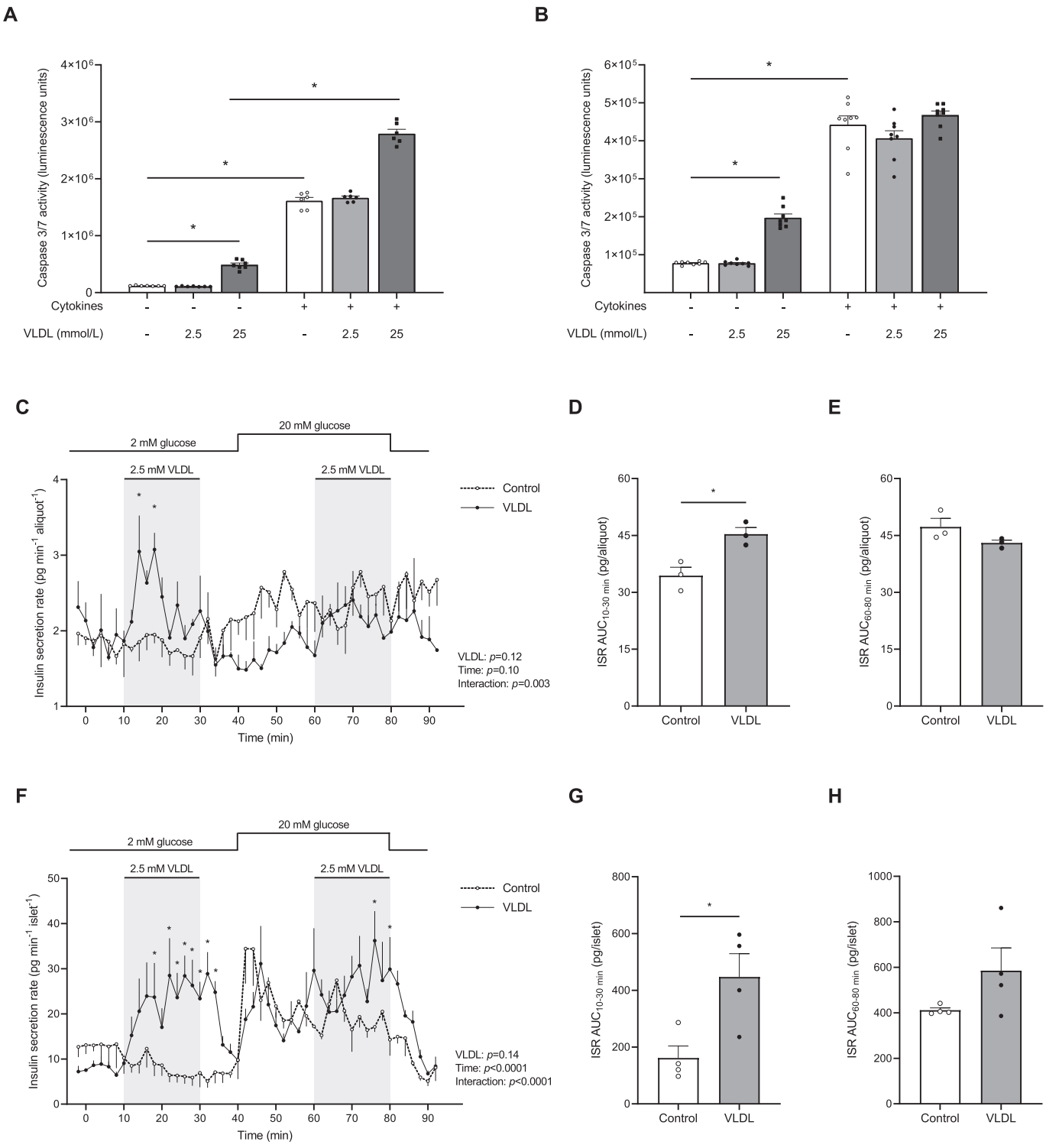

Fig. 4. Dynamic insulin secretion in response to high physiological triglyceride levels in murine and human pancreatic islets. Basal and cytokine-induced apoptosis assessed by caspase activity in mouse clonal MIN6 β cells exposed for 24 h (A) or 72 h (B) to high physiological (2.5 mmol/L) or supraphysiological (25 mmol/L) VLDL concentrations. Basal (2 mmol/L glucose) and glucose-stimulated (20 mmol/L glucose) dynamic insulin secretion in MIN6 pseudo-islets (C-E) and human pancreatic islets (F-H) exposed to high physiological (2.5 mmol/L) VLDL concentrations assessed by perifusion studies. The areas under the curve of insulin secretion under basal conditions (ISR $AUC_{10–30}$) and glucose-stimulated conditions (ISR $AUC_{60–80}$) were calculated by the trapezoidal rule in MIN6 pseudo-islets (D and E) and human islets (G and H). Group means ± SEM and individual values are shown. Differences were analyzed using Kruskal-Wallis test followed by Dunn's *post hoc* pairwise comparisons when appropriate. Repeated measures were analyzed by two-way ANOVA including study condition (VLDL *vs* control), time, and study × time interaction effects as factors. If this test revealed statistically significant effects, multiple pairwise comparisons were performed using Tukey's *post hoc* tests. * $P < 0.05$.

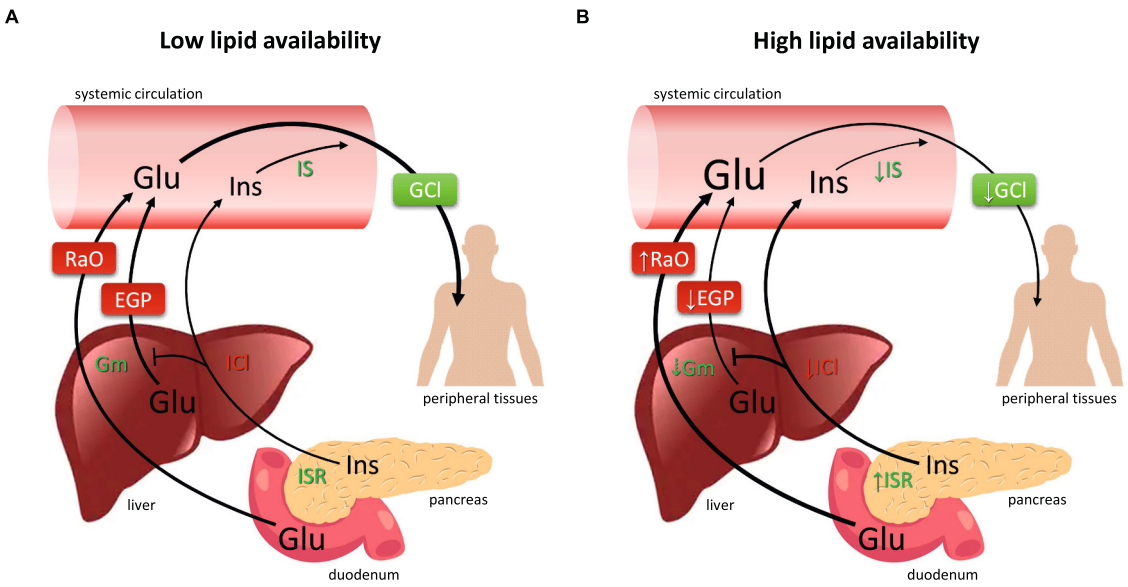

Fig. 5. Schematic overview of glucose homeostatic mechanisms at baseline (A) and during acute hypertriglyceridemia (B). Mechanisms lowering and increasing plasma glucose concentrations are represented in green and red, respectively. Line weights and vertical arrows represent relative changes induced by acute hypertriglyceridemia. The dotted arrow indicates a speculative mechanism.
Abbreviations: EGP, Endogenous Glucose Production; GCl, Glucose Clearance; Glu, Glucose; Gm, splanchnic Glucose metabolism; ICl, Insulin Clearance; Ins, Insulin; IS, Insulin Sensitivity; ISR, Insulin Secretion Rate; RaO, Rate of appearance of Oral glucose. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mild enhancement in β cell potentiation, a variable that captures the physiological property of the β cell to secrete more insulin in the later than earlier phase of the OGTT [24,35].

To our knowledge, the direct effect of high VLDL concentrations on β cell viability and function has been previously investigated only in a single study *in vitro* [34]. In that study, exposure of clonal β cells and mouse islets to triglyceride-rich lipoproteins increased apoptosis and reduced the level of insulin transcript in a dose- and time-dependent fashion. Notably, the authors used human lipoproteins on mouse cells and pancreatic islets and compared the effect of low *vs* supra-physiological lipoprotein levels, considering the normal range observed in humans. Thus, the effect of high-normal lipoprotein levels on human β cells remained unknown. In our *in vitro* experiments, we observed that β cell apoptosis is not affected by prolonged exposure to high triglyceride-rich VLDL concentrations within the physiological range. In keeping with these finding, it has been demonstrated that high triglyceride levels *per se* are not a causative factor for loss of β cell mass in humans [36] and mice [37] with genetic predisposition to hypertriglyceridemia, in the absence of hyperglycemia and insulin resistance. These findings are also partially in agreement with the study from Roehrich et al. [34], showing that VLDL exposure induces β cell apoptosis and reduces insulin transcription at supraphysiological lipoprotein concentrations, but is neutral at low-physiological VLDL levels. Using an elaborate perifusion protocol to detail the dynamic insulin secretory responses to changes in VLDL availability with high time resolution, we demonstrated that high-physiologic VLDL levels rapidly increase β cell insulin secretion at basal glucose levels in both murine β cells and human pancreatic islets, whereas they increase glucose-stimulated insulin secretion only in human pancreatic islets. Noteworthy, the ISR returned to baseline values shortly after VLDL infusion was suspended under both glucose conditions, suggesting that increased insulin concentrations in the perifusate fluid were the result of lipid-induced insulin secretion and not of insulin release from damaged β cells. This newly generated evidence does not support a short-term lipotoxic effect of hypertriglyceridemia on β cell mass and function. Conversely, these findings align with the hypothesis of a causal relationship between high-physiological triglyceride availability and insulin hypersecretion supported by previous observational studies [19,20]. A plausible explanation for the divergence between clinical and experimental findings with regard to β cell function may relate to different glucose levels achieved (more extreme in *in vitro* studies) and/or to different local concentrations of triglyceride-rich lipoproteins at the β cell level when triglycerides are supplemented by *i.v.* infusion or directly into the culture medium. In fact, though changes in circulating VLDL levels affect lipoprotein concentration in the interstitium, a 5.5-fold concentration gradient between plasma and interstitial fluid has been documented [38]. Moreover, sustained hyperinsulinemia has been demonstrated to diminish insulin secretion in response to hyperglycemia in healthy young subjects [39]. This negative feedback would be absent in perifusion experiments, wherein the medium is constantly replaced.

Lipid-induced hyperinsulinemia in our clinical study was driven, besides glucose-dependent insulin hypersecretion, by reduced insulin clearance at fasting and during the OGTT, which allows a greater proportion of the secreted insulin to reach the systemic circulation. Previous studies reported that insulin clearance was reduced [16] or not affected [14] by lipid infusion. Conflicting results may be explained by different populations and techniques to estimate insulin clearance in humans, for which a gold-standard method of measurement is still to be established [40–42]. The liver is the primary site of endogenous insulin extraction, accounting for the majority (up to 80 %) of secreted insulin removal during its first-pass transit into the portal system [43]. Although a wide interindividual variability exists, hepatic insulin clearance is typically reduced in insulin-resistant obese individuals [41,44,45]. This has been traditionally regarded as a compensatory mechanism for impaired insulin sensitivity to allow a larger fraction of the secreted insulin to reach the systemic circulation. In the long term, however, this process may lead to chronic hyperinsulinemia [44], which has been identified as a key pathophysiological factor in the etiology of diabetes [46–48].

The use of stable glucose isotopes during the OGTT allowed us to accurately measure the three major metabolic processes governing glucose homeostasis (namely GCl, EGP and RaO), all of which were affected by lipid infusion and contribute to our understanding of lipid-induced glucose intolerance and insulin resistance. We found that absolute GCl, a marker of glucose uptake and utilization by peripheral

tissues, was reduced by hypertriglyceridemia despite higher insulin levels. A marked deterioration of whole-body insulin sensitivity, contributing to hyperglycemia, was confirmed when GCl was corrected by insulin levels. Conversely, EGP, which mostly occurs in the liver, was more suppressed during the OGTT when lipids were infused, opposing to hyperglycemia. Given that glucagon levels were unchanged (Fig. 1M), higher insulin and glucose levels can explain the unexpected reduction of EGP. In fact, the profiles of EGP adjusted for ISR during saline and lipid infusion were virtually identical over the first 90 min of the OGTT, when the suppression of EGP occurred (Supplemental Fig. 1). Previous studies reported that a lipid infusion had neutral [17] or even negative [12,13] effects on the physiological suppression of EGP during a glucose challenge, which was explained by the greater glycerol availability and hepatic insulin resistance. In our study conditions, however, a marked increase in glycerol levels is unlikely (*i.e.*, minor rise in FFA compared with the 3-fold increase observed in similar previous studies [13]) and we did not observe hepatic insulin resistance at fasting (EGP × FPI) and during the OGTT (EGP × ISR). The notion that peripheral but not hepatic insulin resistance is primarily affected by hypertriglyceridemia, at least when it is not accompanied by large increments in FFA, is novel and deserves further investigation. Additionally, we also found that RaO was higher during the early phase of the OGTT when lipid was infused. A growing body of evidence demonstrates that RaO is a main determinant of postprandial glucose peak and overall glucose tolerance in non-diabetic individuals [26,49]. RaO can be modulated by two physiological mechanisms: intestinal glucose absorption rate, which in turn is regulated by the gastric emptying rate, and splanchnic glucose uptake (intended as first-pass glucose removal in the splanchnic region before it reaches the inferior vena cava). Therefore, a definite mechanistic explanation for the increased RaO would require direct measures of gastric emptying, intestinal glucose absorption, and splanchnic glucose uptake, which were not available in this study. An increased RaO was observed also in a previous study using a similar double-tracer technique and employing a higher lipid infusion rate [13]. In that case, it was attributed to the recycling of the ^{13}C from labeled oral glucose through the gluconeogenic pathway in the later phases of the OGTT, possibly stimulated by the relative abundance of FFA [13]. This explanation however does not fit our data, showing a lower FFA increase, reduced EGP, and higher RaO in the first rather than last part of the OGTT. In this healthy population, acute hypertriglyceridemia has an almost neutral effect on total glucose rate of appearance for its opposite actions of similar magnitude on EGP and RaO. This effect, however, may be more clinically relevant in conditions where EGP and RaO might be differentially affected by triglyceride availability (*e.g.* in the presence of insulin resistance).

Some limitations of our human and *in vitro* studies should be considered. Though the slow lipid infusion rate and the avoidance of heparin co-infusion limited the rise in circulating FFA, we cannot exclude that the clinical effects of triglycerides are at least in part mediated by higher FFA concentrations resulting from triglyceride metabolism. Furthermore, the two infusions were not isocaloric and this might have influenced the study results. The repeated labeled OGTT, designed to evaluate simultaneously glucose tolerance and major glucose homeostatic mechanisms, represents a more physiological but less controlled condition compared with "gold standard" methods based on glucose clamps. Glucose tracer data show slower glucose absorption during lipid infusion in the later phase of the OGTT, in contrast to the first 120 min. However, the incomplete recovery of the oral glucose load in the 180-min observation period does not allow to establish the overall effect of acute hypertriglyceridemia on RaO. During both lipid and saline infusions, EGP and GCl patterns showed, respectively, a late increase and an early suppression that were more pronounced compared with previous studies [50]. As we compared the effects of the two infusions, differences with other studies, which may be attributed to different subjects' characteristics, should not affect the interpretation of our findings. The clinical study was conducted in healthy, young, lean Caucasians. Thus, study findings may not apply to other populations with concomitant metabolic alterations that may be more susceptible to the effects of triglycerides on insulin secretion and action, including insulin resistant patients with obesity and/or type 2 diabetes, elderly people, and individuals with an unfavorable genetic and ethnic background. In our study, women are under-represented as we unintentionally recruited almost all men. Given the known metabolic differences between the two sexes [51], our findings should be confirmed in a larger group of women. Also, we increased plasma triglyceride concentrations by an *i.v.* lipid infusion; post-prandial hypertriglyceridemia resulting from oral lipid consumption may have different effects on glucose tolerance [52]. The confirmation of findings obtained in murine β cells in pancreatic human islets represents a major strength of our *in vitro* study, given the known differences between the two species and the greater responsiveness to glucose and non-glucose stimuli of human islets [53]. While we used an elaborate perifusion protocol to quantify the dynamic insulin secretory responses of β cells, longer-term experiments are still required to examine the chronic effect of VLDL on human β cell viability and insulin secretion. The molecular mechanisms involved in lipotoxicity and insulin secretion have been not examined, so further research is required to elucidate lipid-induced changes in gene expression and metabolic pathways within β cells and peripheral tissues.

5. Conclusions

In conclusion, we have demonstrated that mild acute hypertriglyceridemia impairs glucose tolerance in healthy lean subjects by inducing insulin resistance, β cell dysfunction, and enhanced RaO, which are only partly compensated by the hyperglycemia-driven higher insulin secretion and β cell potentiation. This condition is likely to represent the earliest step of the chain of events leading to diabetes in subjects with dyslipidemia.

CRediT authorship contribution statement

Domenico Tricò: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Visualization, Project administration, Funding acquisition. **Alessandro Mengozzi:** Methodology, Investigation, Data curation. **Simona Baldi:** Methodology, Investigation, Data curation. **Roberto Bizzotto:** Methodology, Software, Data curation, Writing – review & editing. **Oladapo Olaniru:** Methodology, Investigation. **Klaudia Toczyska:** Methodology, Investigation. **Guo Cai Huang:** Methodology, Investigation. **Marta Seghieri:** Methodology, Investigation. **Silvia Frascerra:** Methodology, Investigation, Data curation. **Stephanie A. Amiel:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision. **Shanta Persaud:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision. **Peter Jones:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision. **Andrea Mari:** Methodology, Software, Data curation, Writing – review & editing. **Andrea Natali:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2022.155247.

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