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Postprandial insulin action relies on meal composition and hepatic parasympathetics: dependency on glucose and amino acids
Meal, parasympathetics & insulin action

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Abstract

Insulin sensitivity (IS) increases following a meal. Meal composition affects postprandial glucose disposal but still remains unclear which nutrients and mechanisms are involved. We hypothesized that gut-absorbed glucose and amino acids stimulate hepatic parasympathetic nerves, potentiating insulin action.

Male Sprague–Dawley rats were 24 h fasted and anesthetized. Two series of experiments were performed. (A) IS was assessed before and after liquid test meal administration (10 ml.kg^{-1}, intraenteric): glucose + amino acids + lipids (GAL, n=6); glucose (n=5); amino acids (n=5); lipids (n=3); glucose + amino acids (GA, n=9); amino acids + lipids (n=3); and glucose + lipids (n=4). (B) Separately, fasted animals were submitted to hepatic parasympathetic denervation (DEN); IS was assessed before and after GAL (n=4) or GA administration (n=4).

(A) Both GAL and GA induced significant insulin sensitization. GAL increased IS from 97.9±6.2 mg glucose/kg bw (fasting) to 225.4±18.3 mg glucose/kg bw (P<0.001; 143.6±26.0% potentiation of IS); GA increased IS from 109.0±6.6 to 240.4±18.0 mg glucose/kg bw (P<0.001; 123.1±13.4% potentiation). None of the other meals potentiated IS. (B) GAL and GA did not induce a significant insulin sensitization in DEN animal.

To achieve maximal insulin sensitization following a meal, it is required that gut-absorbed glucose and amino acids trigger a vagal reflex that involves hepatic parasympathetic nerves.

Keywords: Insulin action; Insulin sensitivity; Meal composition; Parasympathetic nerves; Protein intake; Glucose; Nutritional modulation of insulin sensitivity

1. Introduction

Glucose homeostasis comprises several features, being insulin-dependent glucose disposal is the most complex and pertinent. This is particularly true during food intake, when gut-derived blood glucose excursions increase, requiring higher insulinemia and insulin action to restore basal glycemia. Accordingly, more attention has been given to the postprandial state, since in the course toward diabetes, deregulation of glucose homeostasis occurs in the postprandial prior to the fasted state [1–4].

The ingestion of a meal leads to the rise of plasma glucose, which stimulates pancreatic β-cells to release insulin, a mechanism potentiated by incretin hormones, namely *glucagon-like peptide 1* (GLP-1) and gastric inhibitory peptide (GIP) [5]. Postprandially, there is also parasympathetic stimulation, particularly of the hepatic branch [6,7], which induces hepatic nitric oxide production that, along with hepatic glutathione, is essential to achieve maximal insulin action in peripheral organs [7–13], namely *skeletal muscle*, *kidney* and *heart* [14], but not in liver, pancreas and adipose tissue. Hepatic parasympathetic impairment, as well as inhibition of nitric oxide or glutathione syntheses, was shown to decrease postprandial insulin action to levels observed in the fasted state [4,7,13,15,16].

Knowing that insulin sensitivity increases following a meal leads us to the question on how nutritional composition affects such mechanism. It is widely recognized that meal composition has a significant impact on postprandial glucose disposal, both acutely and chronically [4,8,16–21]. However, the precise role of each class of nutrients in peripheral insulin action is poorly understood. To address this question, our group has previously observed that, unlike mixed-meals, carbohydrates alone do not seem to be an adequate feeding signal to increase insulin action [8], suggesting that carbohydrates do not adequately mimic the effects of regular mixed-meals [8]. The precise nutrients involved in insulin sensitization, their significance and mechanism of action remained undetermined.

The purpose of the present work was to explore which nutrients are required to trigger a maximal meal-induced insulin sensitization. Thus, we present a systematic study on how different nutrients affect

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postprandial insulin action. Our hypothesis was that only a meal containing both glucose and amino acids (A) is proficient to increase peripheral insulin action through a mechanism dependent on hepatic parasympathetic activation but independently of insulin secretion. It is proposed for the first time an explanatory mechanism for the meal-induced insulin sensitization.

2. Methods

2.1. Animals and surgical procedures

Concerning ethical animal use, we followed the Principles of Laboratory Animal Care (NIH Publication 85-23, revised 1985) and the European guidelines forthe protection of animals used for scientific purposes (European Union Directive 2010/63/EU).

We used 9-week-old male Sprague–Dawley rats (Charles River, Barcelona, Spain), maintained in a 12:12 h light–dark cycle, with free access to food and water.

Animals were submitted to a 24-h fasting period, starting the day before the experiment. Animals were anaesthetized using sodium pentobarbital (65 mg/kg, intraperitoneal). Trachea, right femoral artery and vein were surgically cannulated to place an external arterial–venous shunt for arterial blood sampling, intravenous (iv) drug infusion and blood pressure monitoring (ML750, ADInstruments, CO, USA), as described [22]. An intestinal band was placed (0.5 cm downstream from pyloric sphincter) to avoid stomach–gut communication, and enteric cannulation was performed (1 cm downstream from pylorium) for intraenteric administration. Either denervation (DEN) of the hepatic anterior plexus (denervated groups) or sham surgery was performed as described [8]. Anesthesia was maintained by pentobarbital continuous infusion (10 $mg.h^{-1}.kg^{-1}$, iv). Body temperature was kept at 37.0±0.5°C (homeothermic control unit, Harvard Apparatus, Hollinston, MA, USA). Following surgery, a 30-min period was allowed for stabilization.

The route chosen to deliver the meals was the intraenteric instead of gavage, mainly because previous experiments from our group suggested that intragastric and intraenteric administrations produce different insulin-dependent glucose disposal (Afonso and Macedo, unpublished results), and also to overcome the effect of gastric emptying. On the other hand, anesthesia interferes with peristaltism and gastric emptying, which consequently affects food delivery to the intestine, making it impossible to assure a precise meal dosage. Full absorption is not complete during the course of the postmeal rapid insulin sensitivity test (RIST).

2.2. Test meals composition

The seven liquid test meals used in this work were obtained by different combinations of glucose, amino acids and lipids: glucose (G, n=5), lipids (L, n=3), amino acids (A, n=5), glucose + amino acids (GA, n=9), glucose + lipids (GL, n=4), amino acids + lipids (AL, n=3), or glucose + amino acids + lipids (GAL, n=6).

The meals formulation was based on our previous report on the effect of a commercially available diet (Boost, Mead Johnson Nutritionals, Canada) [8,23].

Glucose (1.730 g/kg) was provided by D-glucose >99.5% (Sigma–Aldrich, Lisboa, Portugal); A (4 ml/kg) source was an amino acid iv solution (Aminoplasmal Hepa 15N/l, BBraun; Germany); L (1.910 ml/kg) were provided by medium- and long-chain triglycerides iv solution (Lipofundin MCT/LCT 20%, BBraun; Germany). Test meals were prepared in water (10-ml final volume).

2.3. Insulin sensitivity assessment

As mentioned above, peripheral insulin sensitivity was assessed by two methods, the RIST and the intravenous insulin tolerance test (ivITT).

RIST is a modified euglycemic clamp that allows quantification of insulin-dependent glucose uptake specifically by peripheral tissues in both fasted and fed states (in the same animal), while inhibiting hepatic glucose production [11,16,24]. Insulin sensitivity is determined by the total amount of glucose infused to maintain euglycemia (RIST index, mg glucose/kg body weight, bw) upon a 50-mU/kg insulin administration [16].

ivITT, a widely accepted method to assess insulin sensitivity both in the fasted and in the fed stat [25,26], was the second method used to determine insulin sensitivity. As previously described [25], the decline of arterial glycemia following an insulin bolus (100 mU/kg, iv) was measured, a linear regression of the glycemia decline was performed and its slope was used to calculate the constant rate of glucose disappearance (K_{ITT}, % glucose/min). K_{ITT} <2 corresponds to insulin resistance.

2.4. Plasma glucose, insulin, C-peptide, GIP and GLP-1 levels

Glycemia was determined by the glucose oxidase method (1500 Sport Glucose Analyzer, Yellow Springs Instruments, USA), in the fasted state, before and after each insulin sensitivity assessment and after test meal administration (t=0, 5, 10 15, 20, 30, 45, 60, 75, 90, 100, 110, 120 min).

Plasma insulin and C-peptide were measured at baseline (before fasting RIST), immediately before administration of the test meal (t=0 min), after the test meal (t=15, 30, 60, 100, 120 min) and after postmeal RIST. Plasma incretin (GLP-1 and GIP) levels were determined before (t=0 min) and after meal administration (t=30 min and t=120 min). Plasma insulin, C-peptide, GIP and GLP-1 concentrations were determined by ELISA [Mercodia Rat Ultrasensitive Insulin ELISA; Mercodia Rat C-peptide ELISA, Mercodia AB., Uppsala, Sweden; Rat/Mouse GIP (Total) ELISA kit; GLP-1 Total ELISA kit, Millipore, St. Charles, USA; respectively].

2.5. Experimental protocols

Two series of experiments were performed.

First, we studied the effect of meal composition on postprandial insulin sensitivity, using the RIST methodology. Following surgery, fasting arterial glycemic baseline was determined, and an initial insulin sensitivity assessment was made (control RIST). The liquid test meal was given intraenterically (10 ml/kg, 60 ml/h, IE), and 2 h afterwards, a new insulin sensitivity assessment was made (postmeal RIST). Potentiation of insulin action was determined by the percentage of RIST index increment after each of the seven test meals. In a separate set of animals, ivITT methodology was used to further compare fasting insulin sensitivity (24 h fasted, n=3) with insulin sensitivity obtained 2 h after administration of either G (n=4) or GA (n=3) meals (10 ml/kg, 60 ml/h, IE).

The second series of experiments aimed to determine the relevance of hepatic parasympathetic nerves in the insulin action increment after a meal. For that, one set of animals with previous surgical DEN of the hepatic anterior plexus was submitted to a RIST in the 24-h fasted state (control RIST), followed by a second RIST 2 h after either GAL (GAL-DEN, n=4) or GA (GA-DEN, n=4) administration (10 ml/kg, IE) — postmeal RIST. In an additional set of animals, ivITT was also used to assess insulin sensitivity in previously hepatic-denervated animals receiving either G (G-DEN, n=3) or GA (GA-DEN, n=3).

2.6. Statistical analyses

Data are presented as means±S.E.M. Significant differences were calculated using the following approaches: two-tailed Student's t tests, to compare fasting with postmeal insulin sensitivity (evaluated in the same animal); and one-way ANOVA followed by Tukey–Kramer multiple comparison tests, to compare different groups of animals. GraphPad Prism 5.01 software (GraphPad Software, La Jolla, USA) was

used for the statistical analyses. Differences were acceptedas significant at $P<0.05$.

3. Results

3.1. Basal parameters

Body weight, blood pressure, plasma glucose, insulin, C-peptide, GLP-1 and GIP were evaluated in the basal state. There were no significant differences in basal fasting parameters between test groups (Supplementary Table 1).

3.2. Insulin sensitivity

Fasting insulin sensitivity was similar among all groups. GAL and GA meals induced significant increase in insulin action ($P<0.001$ *vs.* fasting insulin sensitivity), whereas none of the other meals (G, A, L, GL, AL) affected insulin action (Fig. 1). Accordingly, postmeal insulin sensitivity was higher after GAL and GA when compared with the other test meals (Fig. 1).

By calculating the percentage increment of insulin sensitivity after a meal in comparison with the fasting insulin sensitivity, we determined the potentiation of insulin sensitivity (%) induced by each test meal, which allows us a direct comparison of the different test meals capacity to ensure a higher insulin action in the postprandial state. Higher insulin sensitivity potentiation implies a better ability to regulate glucose homeostasis after a meal. As shown in Fig. 1, potentiation of the fasting insulin sensitivity was significantly higher following GAL and GA (GAL, 143.6±26.0 %; GA, 123.1±13.4%) than after the other test meals (G, 37.8±13.8%, $P<0.01$; A, 22.8±14.7%, $P<0.001$; L, 16.0±2.3%, $P<0.01$; GL, 41.2±5.5%, $P<0.05$; AL, 47.6±14.2%, $P<0.05$; *vs.* GAL and GA).

The ivITT set of experiments confirmed that administration of a test meal containing glucose and amino acids (GA) results in higher insulin sensitivity than that observed in either fasting or post-glucose meal (G) states (Fig. 2). Indeed, K_{ITT} was higher in the GA group (5.03±0.12% glucose/min) than in both fasted (fasting K_{ITT}, 2.92±0.25% glucose/min; $P<0.01$) and glucose meal (K_{ITT}, 2.94±0.22% glucose/min; $P<0.001$), with no significant differences between the last two groups, suggesting that GA meal induces higher insulin sensitivity than G meal.

To assess the involvement of the hepatic parasympathetic nerves on meal-induced insulin sensitization, hepatic parasympathetic DEN was performed before GAL (GAL-DEN) and GA (GA-DEN). Hepatic DEN prevented meal-induced insulin sensitization following intraenteric administration of either GAL or GA meals (Fig. 3). In the denervated animals, peripheral insulin sensitivity did not increase following meal administration, either in GAL-DEN (fasting RIST, 99.0±18.6-mg glucose/kg bw; postmeal RIST, 123.8±34.5-mg glucose/kg bw; ns; Fig. 3A) or GA-DEN group (fasting RIST, 116.8±6.0-mg glucose/kg bw; postmeal RIST, 132.5±7.2-mg glucose/kg bw; ns; Fig. 3B).

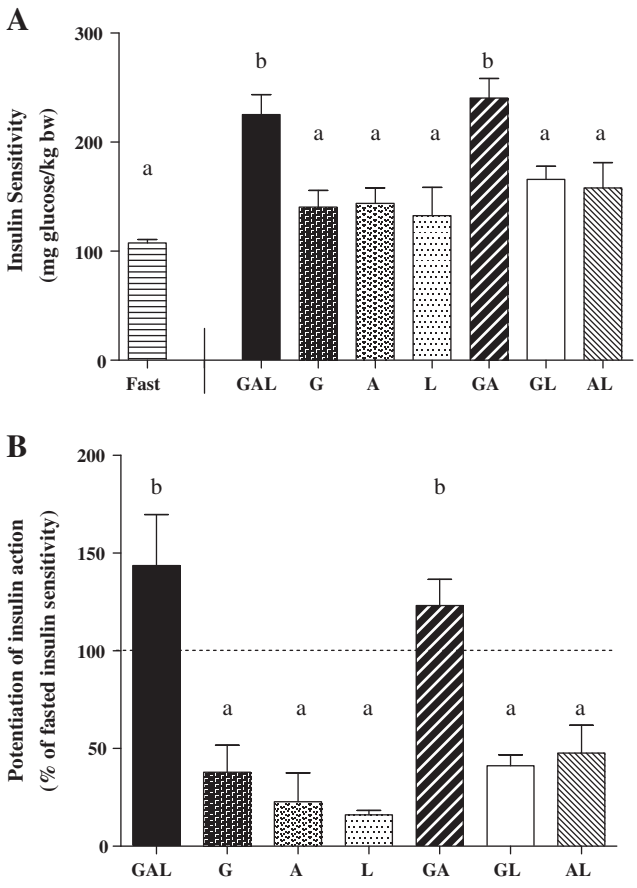

Fig. 1. Effect of the different test meals on fasting insulin sensitivity. (A) Fasting and postmeal insulin sensitivity. GA and GAL significantly increase insulin sensitivity from the fasted state, unlike the other test meals. (B) Potentiation of insulin action induced by each of the meals tested. GAL and GA meals more than doubled insulin action observed in the fasted state (>100% potentiation), whereas the other test meals did not induce significant changes from the fasted state. This suggests that glucose and A are required to trigger the meal-induced insulin sensitization. GAL ($n=6$); G ($n=5$); A ($n=5$); L ($n=3$); GA ($n=9$); GL ($n=4$); AL ($n=3$). Conditions not sharing the same superscript letter differ significantly ($P<0.05$; one-way ANOVA followed by Tukey multiple comparisons test).

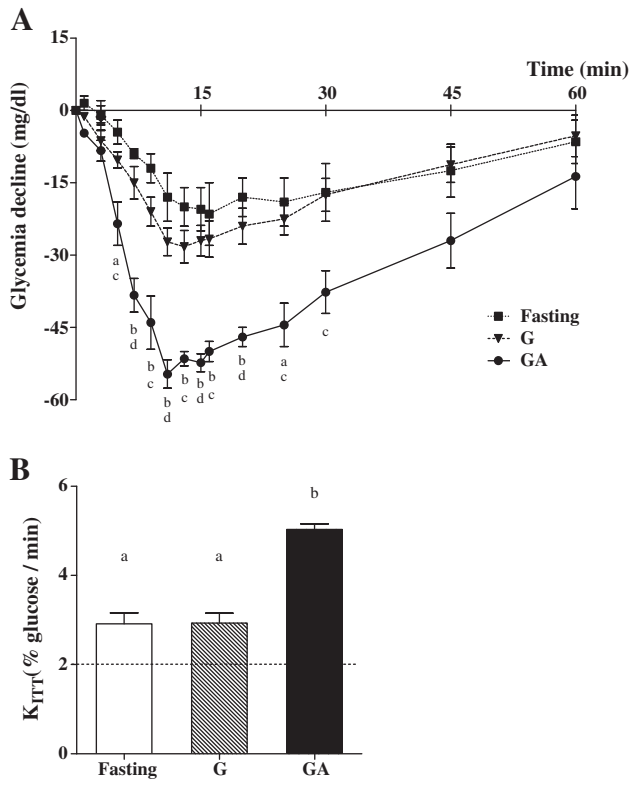

Fig. 2. Postprandial insulin sensitivity following a meal composed of GA is higher than insulin sensitivity in the fasted state or G, as assessed by the ivITT. (A) Glycemia decline (normalized to basal glycemia) following insulin bolus (t=0 min) is more pronounced and sustained in the GA group (circles) than in both fasting (squares) or G group (triangles); one-way ANOVA followed by Tukey at each time point: $^{a}P<0.05$, $^{b}P<0.01$ (*vs.* fasting); $^{c}P<0.05$, $^{d}P<0.01$, $^{e}P<0.001$ (*vs.* G). (B) The constant rate of glucose disappearance (K_{ITT}), used to quantify insulin sensitivity, was significantly higher in the GA (filled bar) than in both fasting (blank bar) and G (hatched bar) groups; conditions not sharing the same superscript letter differ significantly ($P<0.05$; one-way ANOVA, Tukey).

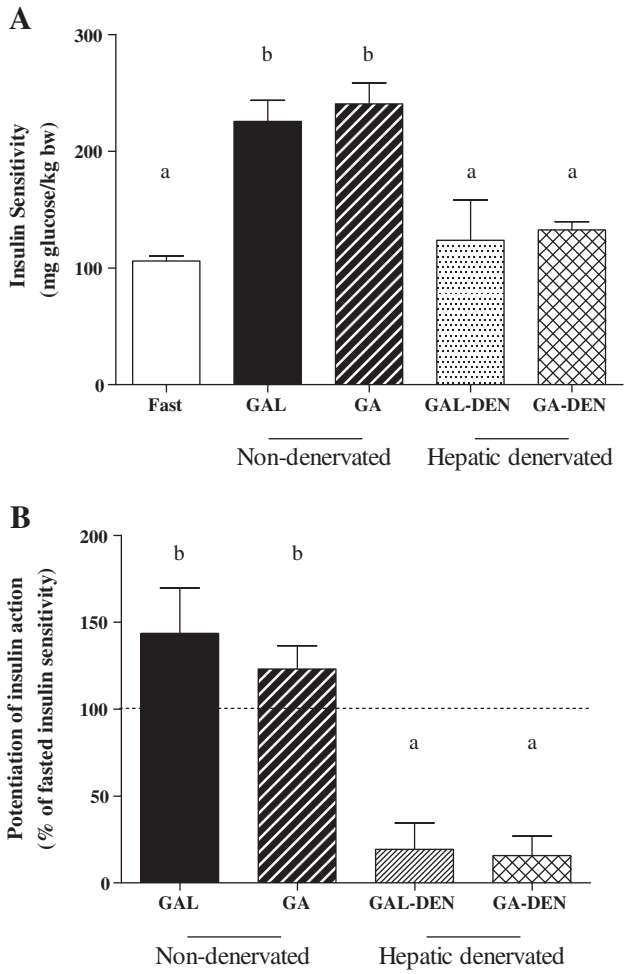

Fig. 3. Hepatic parasympathetic DEN prevents meal-induced insulin sensitization. (A) Increase in insulin sensitivity was observed in sham-operated animals (nondenervated), but not in hepatic-denervated animals (DEN, in the right), either following intraenteric administration of GAL or GA. (B) Potentiation of insulin action following either GAL or GA was significantly impaired in animals with previous hepatic parasympathetic DEN (GAL-DEN and GA-DEN, bars in the right). Conditions not sharing the same superscript letter differ significantly ($P<0.05$; one-way ANOVA, Tukey).

IvITT methodology also showed significantly lower insulin sensitivity (K_{ITT}) in the GA-DEN (2.89±0.29% glucose/min) than in the nondenervated animals (GA, 5.03±0.12% glucose/min; $P<0.001$); however, K_{ITT} was not different between G-DEN (3.13±0.32% glucose/min) and G (2.94±0.22% glucose/min) groups.

3.3. Postmeal plasma levels of glucose, insulin and C-peptide

The test meals containing glucose (GAL, G, GA and GL) induced similar glucose excursions during the 120-min after-meal administration, which were significantly higher than those induced by the meals with no glucose present (A, L and AL), as determined by the area under the curves (AUC) of postmeal glycemia profiles (Table 1). The glycemic patterns seemed to be accompanied by both C-peptide and insulin plasma levels, since AUC of both C-peptide and insulin also tended to be higher in the glucose-containing meals than the others (Table 1), although such differences did not reach statistical significance. At the start of postmeal RIST (t=120 min), plasma levels of glucose, insulin and C-peptide were not statistically different between all groups studied.

Table 1
AUC of plasma glucose, C-peptide and insulin, following intraenteric administration of the different test meals

Test meal	Glucose (postmeal AUC, g.dl^{-1}.min)	C-peptide (postmeal AUC, nmol.l^{-1}.min)	Insulin (postmeal AUC, μg.l^{-1}.min)
GAL	20.3±1.1	226.9±29.8	341.5±34.0
G	20.8±1.5	309.1±1.1	318.5±24.9
A	11.8±0.3 [a]	35.2±6.0 [c]	196.9±27.5 [e]
L	12.1±0.8 [a]	195.4±0.1	212.2±11.6
GA	19.6±0.6	183.2±73.6	257.1±42.2
GL	19.7±0.9	330.5±52.5	226.8±33.2
AL	12.7±0.3 [b]	96.0±30.0 [d]	188.3±1.0 [f]

GAL ($n=6$); G ($n=5$); A ($n=5$); L ($n=3$); GA ($n=9$); GL ($n=4$); AL ($n=3$).
[a] $P<0.01$ (*vs.* GAL, G, GA, GL).
[b] $P<0.05$ (*vs.* GAL, G, GA, GL).
[c] $P<0.01$ (*vs.* G, GL).
[d] $P<0.05$ (*vs.* GL).
[e] $P<0.05$ (*vs.* GAL, G, GL).
[f] $P<0.05$ (*vs.* GL).

Concerning the effect of hepatic DEN on glycemia following GAL and GA test meals (Fig. 4), no significant differences were found between sham and denervated groups (GAL-DEN and GA-DEN), either in terms of postmeal glycemia profile or AUC (GAL, 20.3±1.1 g.dl^{-1}.min; GA, 19.6±0.6 g.dl^{-1}.min; GAL-DEN, 23.0±2.8 g.dl^{-1}.min; GA-DEN, 21.4±0.8 g.dl^{-1}.min; ns), despite the slight tendency for higher glycemia in the denervated animals. In contrast, hepatic-denervated animals presented higher plasma levels of both C-peptide (AUC: GAL-DEN, 460.1±15.5 pmol.l^{-1}.min; GA-DEN, 446.7±18.0 pmol.l^{-1}.min) and insulin (AUC: GAL-DEN, 545.9±73.8 μg.l^{-1}.min; GA-DEN, 479.3±10.9 μg.l^{-1}.min) than the nondenervated groups (C-peptide: GAL, 226.9±29.8 pmol.l^{-1}.min, $P<0.05$; GA, 183.2±73.6 pmol.l^{-1}.min, $P<0.05$; insulin: GAL, 341.5±34.0 μg.l^{-1}.min, $P<0.05$; GA, 257.1±42.2 μg.l^{-1}.min, $P<0.05$) (Fig. 4). The increase in insulin secretion observed in hepatic-denervated animals seems to be due to a higher β-cell function capacity observed in these animals (Fig. 4G), in what seems to be a compensatory mechanism to avoid higher glucose excursions.

3.4. Effect of meal composition and hepatic DEN on plasma incretin levels

Both GLP-1 and GIP increased from the fasting to the postmeal state in all groups, within 30 min after the meal (Fig. 5). The trend for higher GLP-1 levels in GAL and GA groups was confirmed by the 120-min AUC following the meals. GLP-1 AUC was higher in both GAL (5.3±0.2 nmol.l^{-1}.min) and GA (5.2±0.2 nmol.l^{-1}.min) than in G (4.1±0.4 nmol.l^{-1}.min; $P<0.05$) and A (3.7±0.4 nmol.l^{-1}.min; $P<0.05$) groups. No significant differences were found in postmeal GIP AUC between groups (Fig. 5).

Comparison between hepatic parasympathetic-denervated (GAL-DEN, GA-DEN) and nondenervated (GAL, GA) animals showed a trend for higher incretin levels in the denervated group (Fig. 5), in particular for GLP-1 (postmeal AUC: GAL-DEN, 8.1±0.2 nmol.l^{-1}.min; GA-DEN, 7.3±0.7 nmol.l^{-1}.min; $P<0.05$ *vs.* nondenervated). These data are compliant with the higher insulin production (Table 1) in the hepatic-denervated animals to prevent exacerbated glucose excursion due to the impaired meal-induced insulin sensitization.

4. Discussion

The results reported herein suggest that both glucose and A are required in the intestine to trigger insulin sensitization that occurs in the postprandial state, independently of insulin secretion. This increase in insulin sensitivity requires hepatic parasympathetic activation, impairment of which ultimately results in postprandial insulin resistance.

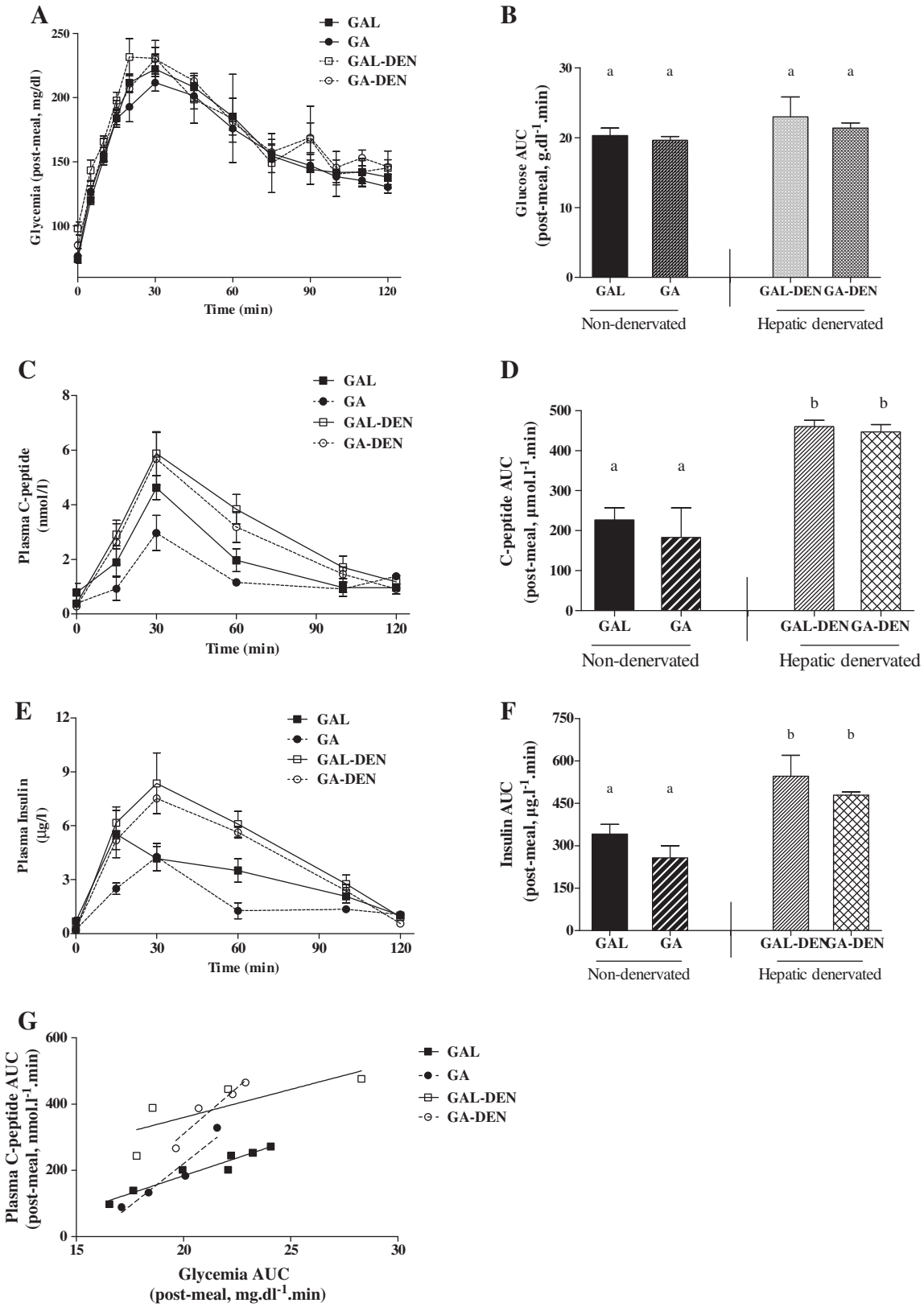

Fig. 4. Profiles and AUC of plasma levels of glucose (A, B), C-peptide (C, D) and insulin (E, F), following administration of either GAL or GA, in hepatic parasympathetic denervated and sham-operated rats. Both C-peptide and insulin plasma levels are higher in the denervated than nondenervated animals, explaining the similar glucose profiles observed. Accordingly, evaluation of β-cell function capacity revealed that β-cell function capacity is higher in both hepatic-denervated animals (G), as given by the linear regression slopes of postmeal plasma C-peptide AUC × glycemia AUC. Conditions not sharing the same superscript letter differ significantly ($P<0.05$; one-way ANOVA, Tukey).

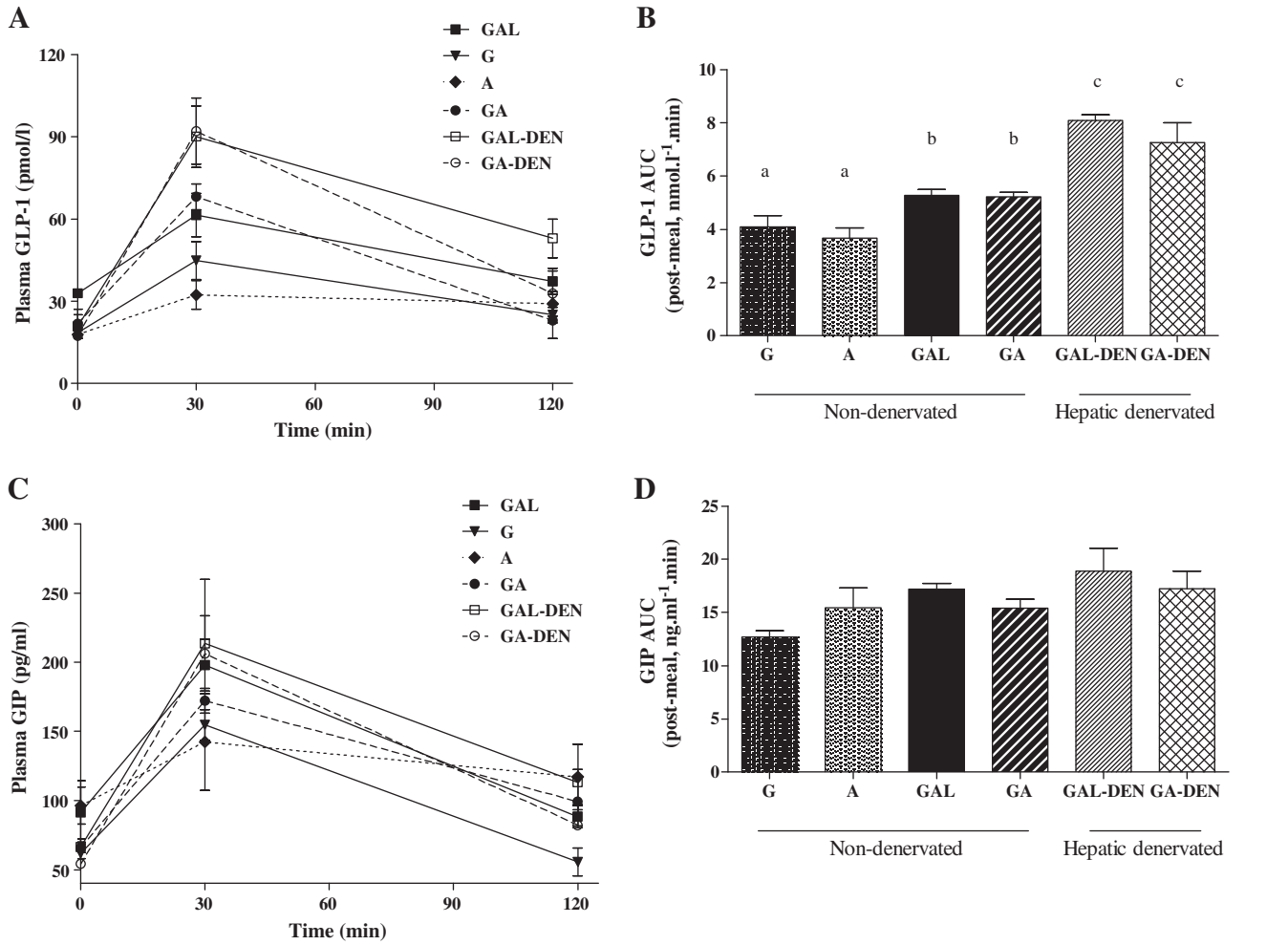

Fig. 5. Plasma levels of GLP-1 and GIP following test meal intraenteric administration tends to be higher in hepatic-denervated (GAL-DEN, GA-DEN) than in sham-operated animals. (A) Plasma profile of GLP-1 for 120 min after the meals. (B) GLP-1 AUC. (C) Plasma profile of GIP for 120 min after the meals. (D) GIP AUC. Conditions not sharing the same superscript letter differ significantly ($P<0.05$; one-way ANOVA, Tukey).

4.1. Postmeal glucose disposal: insulin secretion versus insulin action

To assess the relative influence of each class of nutrients on postmeal plasma glucose disposal, C-peptide, insulin and incretins were assessed after each test meal. As expected, all meals containing glucose resulted in higher glucose excursion, as given by postmeal glycemia AUC. Also, higher glycemia was accompanied by higher C-peptide AUC, as a result of the glucose-induced stimulation of insulin secretion.

A are also known stimulators of insulin secretion [27]. However, when A were given along with glucose, as in GA and GAL test meals, they did not further increase C-peptide. In fact, addition of A to the glucose meals tends to decrease C-peptide, suggesting that A given along with glucose affect glucose disposal by eliciting an increase in peripheral insulin action rather than stimulation of insulin secretion. This increase in insulin action occurs mainly in peripheral organs [7–13], namely *skeletal muscle*, *kidney* and *heart* [14]. This is in accordance with previous observations from others, suggesting that A improve glucose tolerance during an oral glucose tolerance test by a mechanism that does not involve higher insulin secretion [17].

Our data further show that, in acute experiments, insulin secretion does not vary substantially with the meal composition, being proportional to the meal glycemic index. The same is observed for circulating insulin levels, available for peripheral tissues. However, when insulin resistance was induced by surgical ablation of the hepatic parasympathetic nerves, as previously described [7,16,28,29], there was an increase in pancreatic β-cell function capacity, along with higher plasma C-peptide and insulin, without any changes in glycemia, suggesting that insulin secretion constitutes a compensatory mechanism for impaired insulin action in the insulin resistant (*i.e.*, denervated) animals.

To further investigate this mechanism of compensatory insulin secretion, we assessed postprandial plasma incretin levels (GIP and GLP-1). Meal administration elicited a rise in both GIP and GLP-1. As expected, the meals inducing higher plasma GLP-1 were the ones containing glucose, since GLP-1-stimulated insulin secretion seems to be dependent on glucose [30]. However, incretin levels were significantly higher in the insulin resistant (hepatic-denervated) than control animals, particularly in respect to GLP-1, further suggesting an upregulation of insulin secretion aiming to maintain postprandial glycemia when insulin action is impaired. We were unable to determine if plasma GLP-1 levels were higher due to increased secretion or impaired degradation. Nevertheless, the data presented herein suggest that GLP-1 plays a role on a crosstalk between the intestine, the liver and pancreatic β-cell, in order to regulate insulin secretion and insulin action, although this requires further investigation.

4.2. Effect of meal composition on postprandial insulin action

Our present data show that insulin action more than doubles after either GAL (~144% potentiation) or GA (~123% potentiation), in

contrast with the individual components, none of which induces significant changes in insulin action. Moreover, the sum of insulin action potentiation following glucose, A and L separately (G + A + L ≈ 77%) was approximately half of the potentiation achieved by GAL, which suggests that the mechanism of insulin sensitization following a meal is triggered by a synergistic interaction between nutrients. When combined in pairs to evaluate possible nutrient interactions, it was observed that only GA was capable to increase fasting insulin sensitivity. Thus, although sample size for the lipid group (L) may be somewhat limiting, our data seem to exclude a significant role for L in the process of meal-induced sensitization. These data also reveals that glucose by itself does not mimic an ordinary meal and, therefore, does not trigger the postprandial mechanisms involved in glucose metabolism regulation, corroborating previous statements suggesting that oral glucose testing does not provide the same clinically relevant information as standard mixed meals do [8,31], mainly because glucose solutions do not produce the same physiological adaptations as mixed meals. Our data bring a mechanistic insight to previous observations, both in rats and in humans, suggesting that adding protein to carbohydrates improves regulation of postprandial glycemia [32,33].

The meals caloric content was not taken into account in the present work. Nevertheless, when we compared GAL and GA meals (with different caloric contents), we observed similar effect on insulin sensitization, suggesting that caloric content of the meal is not the main factor involved.

These results suggest for the first time that both glucose and A are required in the gut to trigger the meal-induced insulin sensitization and are consistent with previous reports from our group, showing that hypoglycaemic insulin action increases following intragastric administration of a commercially available meal containing complex carbohydrates, proteins and L, in addition to several other unknown components [8]; precisely which classes of nutrients are involved in this process of meal-induced insulin sensitization was a question that remained unanswered at that time. Previously, we have demonstrated that the increase meal-induced insulin sensitization occurs specifically in skeletal muscle, kidney and heart [14]. Also in accordance with our present results, others have reported a higher oral glucose tolerance when A were given along with glucose, however, without assessing insulin sensitivity and without ruling out the possible role of gut-derived incretins [17]. On the contrary, we demonstrate herein that insulin sensitivity is higher 2 h after a meal composed of glucose and A, independently of GLP-1 effect on insulin secretion.

4.3. Mechanism of postprandial insulin action: role of glucose, A and hepatic parasympathetic nerves

The relevance of autonomic nervous system on glucose homeostasis has long been demonstrated. In particular, the hepatic parasympathetic activation that occurs during meal ingestion [6] is essential for peripheral insulin hypoglycemic action [8,28,29]. Interestingly, human studies revealed that this effect seems to be regulated by insulin itself, since hyperinsulinemia may lead to vagal impairment [34], making it difficult to quantify parasympathetic contribution for insulin action using hyperinsulinemic clamps [34], but allowing us to explain autonomic dysfunction in certain pathological conditions associated with hyperinsulinemia, such as obesity [4]. Likewise, in human nonpathological conditions such as aging, it has been observed that greater longevity is associated with both parasympathetic function [35] and glucose tolerance [36]. Insulin sensitivity has been also shown to depend on parasympathetics in healthy human subjects [9,37], and this relationship is now a target for the treatment-impaired glucose tolerance [38]. Indeed, we observed herein that alteration in the normal parasympathetic activation might impair insulin sensitivity, as observed in the hepatic parasympathetic-denervated animals. Furthermore, not all nutrients stimulate hepatic parasympathetic to the same extent. The meal must contain glucose and A to trigger a mechanism reliant on functional hepatic parasympathetic nerves and, therefore, to fully potentiate insulin-dependent glucose disposal. Only glucose plus A was capable to fully activate the hepatic parasympathetic nerves and concomitantly potentiate insulin action.

Glucose is the major stimulator of insulin secretion. However, alternative roles have been attributed to glucose in glucose homeostasis. One possible role for glucose is to stimulate the hypothalamic MCH neurons, which can be involved in the regulation of peripheral glucose homeostasis [39]. In addition, glucose also stimulates enterochromaffin cells, which release serotonin to activate a vago–vagal reflex involved in the regulation of several gastrointestinal (GI) functions [40,41], one of which is postprandial insulin action [8,11]. This parasympathetic reflex, partially dependent on glucose sensing, is essential for glucose homeostasis, since it is impaired in Type 2 diabetes [42] and could explain the relevance of both glucose and parasympathetics in postprandial insulin action.

In addition to glucose, amino acid content in meals has recently gained relevance in diabetes, either due to their direct effect on glucose tolerance [18,43] or indirect effects such as oxidative stress [44]. Concerning glucose homeostasis, some A are considered to be beneficial [20,43,44], whereas others are believed to be harmful [19]. This dubious perspective of A role on glucose homeostasis was well illustrated by the short review from Gerszten and Wang [45], according to whom branched-chain A can induce insulin resistance, for example, through activation of the mTOR pathway in skeletal muscle, but on the other hand, higher plasma levels of branched-chain A have also been associated with better glucose tolerance [45]. Cysteine (Cys), present in the amino acid meal used herein, is one of the most studied and accepted A in the field of diabetes. Since it is a glutathione precursor, Cys has been studied mostly for to its antioxidant effects and also for its direct beneficial role in improving insulin resistance in animal models [32,46]. The antioxidant effects of Cys/glutathione seem to be important to ameliorate insulin resistance in a chronic perspective. We also demonstrated an acute effect of hepatic glutathione on glucose homeostasis, since its content rises from the fasted to the fed state, which is essential to achieve maximal insulin sensitivity following a meal [13,15]. Recent findings from others further suggest that hepatic glutathione is depleted with aging, which they associate with insulin resistance and seems to be reversed by dietary supplementation with Cys and glycine, two glutathione precursors [47]. Some preliminary experiments from our laboratory using Cys along with glucose indicate that Cys is essential in the meal to allow full potentiation of insulin action (Gaspar and Macedo 2014, unpublished observations).

An additional mechanism for A has been previously described, suggesting that gut-absorbed A lead to serotonin-dependent activation of afferent parasympathetic fibres, producing centrally mediated parasympathetic reflexes that control several GI-related functions [48]. An important branch of the efferent parasympathetic innervation is the hepatic, which is essential to regulate peripheral insulin action after a meal [49]. When hepatic parasympathetic nerves are stimulated following a meal, they induce higher hepatic nitric oxide production, which, along with rise in hepatic glutahione levels, leads to the increase in insulin action, required for adequate postprandial glucose disposal [12,13,15].

Considering the abovementioned and our present data, we propose that glucose and amino acids act synergistically to stimulate enterochromaffin cells in order to trigger a parasympathetic reflex, which is essential for postprandial insulin action. Activation of the efferent hepatic parasympathetic nerves then leads to increased hepatic nitric oxide production. Both hepatic nitric oxide and glutathione, last of which also depends on gut-absorbed A, are required to increase peripheral insulin action.

4.4. Glucose and A: activation of the gut–brain–liver axis?

GI tract, central nervous system and enteric nervous system, the so-called *brain–gut axis*, are involved in a two-way communication

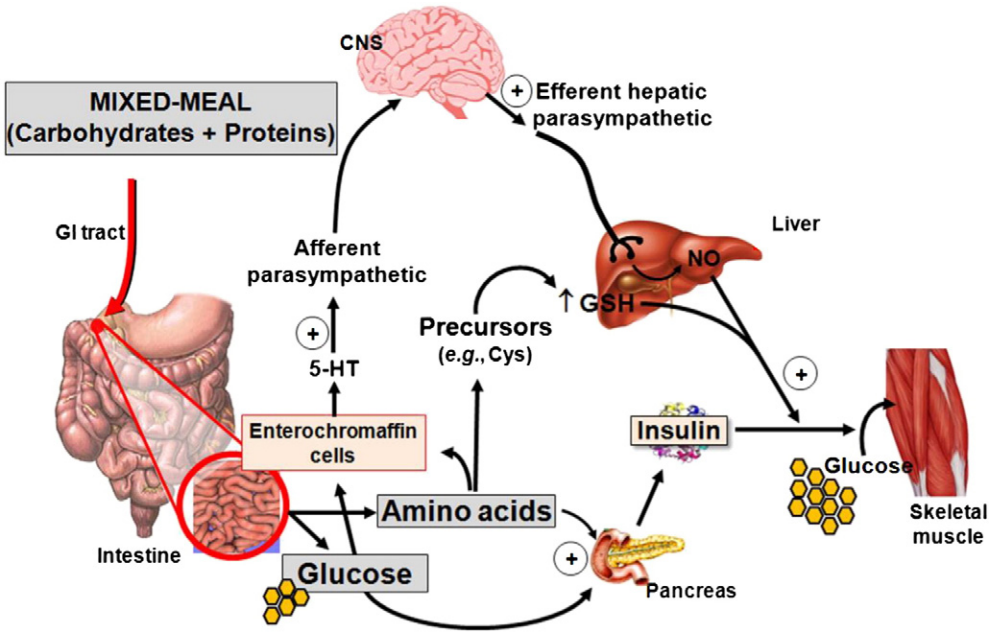

Fig. 6. Working hypothesis for the mechanism of meal-induced potentiation of insulin action. Besides the classic stimulation of the pancreatic insulin secretion, intestinal absorption of glucose and A following ingestion of a mixed-meal containing carbohydrates and proteins triggers a mechanism that results in higher insulin action. The gut-absorbed glucose and A induce the release of serotonin (5-HT) from enterochromaffin cells, which activates parasympathetic afferent terminals and triggers a centrally mediated parasympathetic reflex that results in hepatic nitric oxide (NO) production. Finally, this efferent hepatic parasympathetic-dependent NO, along with increased hepatic glutathione (GSH) synthesis resultant from amino acid absorption, potentiate insulin action in peripheral tissues. 5-HT, serotonin.

that relies on sympathetic and parasympathetic signaling [50,51]. The postprandial effects of this brain–gut axis seem to be mediated by sensorial neurons that induce autonomic reflexes [50]. More recently, the term *gut–brain–liver axis* was introduced to describe a centrally mediated reflex, initiated in the gut and controlling several hepatic functions, namely those related with glucose homeostasis [21], which rely on hepatic vagus nerve [49].

Although those reports did not clearly associate the gut–brain–liver axis with peripheral insulin action, we now propose that gut-absorbed nutrients trigger an autonomic reflex that stimulates hepatic efferent parasympathetic nerves, which along with glutathione, will increase insulin action. This hypothesis is summarized in Fig. 6.

In conclusion, the data presented herein suggest the existence of a mechanism triggered by the presence of glucose plus A in the intestine, which involves hepatic parasympathetic nerves, leading to the rise in insulin-dependent whole-body glucose uptake. This gut-triggered mechanism does not respond to glucose, A or L individually but only to meals containing both glucose and A.

Disclosure

None of the authors have any conflict of interests to declare.

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