Insulin sensitivity is mediated by the activation of the ACh/NO/cGMP pathway in rat liver

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Submitted 20 February 2004; accepted in final form 1 May 2004

The liver has emerged as a key organ in the regulation of peripheral insulin action. A novel hypothesis supports that, in the postprandial state, the rise in blood insulin levels triggers the release of a putative hormone from the liver, which stimulates glucose uptake in skeletal muscle (9).

This putative factor, referred to as hepatic insulin sensitizing substance (HISS), accounts for ~55% of the glucose disposal effect of insulin in the fed state (9, 11).

HISS release is maximal in the postprandial state and decreases with the duration of fasting, leading to a physiological HISS-dependent insulin resistance state. This was demonstrated by testing the response to an exogenous insulin bolus both infasted and fed rats, which showed that the insulin sensitivity in the fasted state is about one-half of that in the postprandial state (11). The feeding signal that triggers HISS action has not been fully clarified.

Even though it is not known if the hepatic parasympathetic nerves and hepatic nitric oxide synthase (NOS) are responsible for the feeding signal, strong evidence supports their involvement in HISS release. This was demonstrated by showing that peripheral insulin resistance can be produced either through hepatic parasympathetic denervation (18, 33, 34), blockade of hepatic muscarinic receptors (31) or of hepatic NOS (4, 25). It was also shown that the insulin resistance resulting from denervation of the liver could be reversed by intraportal (ipv) ACh (32) or a nitric oxide (NO) donor (25). Moreover, insulin resistance caused by intraportal administration of the NOS antagonist L-nitro-arginine methyl ester (L-NAME) is not heightened by atropine, and, similarly, hepatic NOS inhibition does not enhance insulin resistance when induced by hepatic denervation (25).

Notwithstanding the importance of both the hepatic parasympathetic nerves and hepatic NOS in modulating insulin sensitivity through HISS secretion, administration of ACh or of NO donors per se does not enhance peripheral glucose uptake. Insulin is required to disclose HISS action, which suggests that the hepatic parasympathetic nerves and hepatic NOS may play a permissive role in HISS secretion (10).

Binding of ACh to muscarinic receptors leads to NOS activation and NO synthesis (16); however, NO can also stimulate the release of ACh in a number of physiological systems, like the central nervous system or the enteric system (14, 28). Although compelling data demonstrate that ACh and NO are necessary for HISS secretion (9), it is not known whether, in the liver, activation of NOS is a downstream consequence of hepatic parasympathetic nerve stimulation or, in contrast, leads to ACh release and ultimately to HISS secretion.

In addition, the role of NO in HISS release is not well characterized, since there are multiple potential targets for hepatic NO in the HISS synthesis pathway. Ignarro (7) demonstrated that one of the key targets of NO is the heme group of guanylate cyclase (GC). Binding of NO to GC leads to generation of the second messenger cGMP, which is known to be involved in several physiological responses, including vasodilation, inhibition of platelet aggregation, and neuronal...
signaling (7). Therefore, GC emerges as a natural NO target candidate in the HISS pathway.

This study tests the hypothesis that hepatic NO involved in HISS release is synthesized in response to ACh binding to muscarinic receptors in the liver. We compared the ability of ACh and a NO donor, 3-morpholinosidnonimine (SIN-1), to restore HISS action after hepatic NOS antagonism or muscarinic blockade. We also evaluated the role of hepatic GC in HISS release, using the GC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ).

Our results support the hypothesis that ACh triggers hepatic muscarinic receptors, leading to NO synthesis, which, along with GC activation, regulates HISS action.

MATERIALS AND METHODS

Animals

Animals were treated in accordance with the Laboratory Animal Care Guidelines of the European Union (86/609/CEE) and the Institute of Laboratory Animal Resources Guide for Care and Use of Laboratory Animals. Male Wistar rats (8–9 wk) were kept on a 12:12-h light-dark cycle housed one per cage, under temperature control. Rats were allowed ad libitum access to standard rat chow (Panlab A04; Charles River) and tap water. On the day before surgery, the rats were fasted overnight and then allowed access to food for 1 h before the start of the experiment. All experiments began between 9:00 and 10:00.

Presurgical Procedure

Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (65 mg/kg), and anesthesia was maintained throughout the experiment by continuous infusion of pentobarbital sodium (1.0 mg/ml, 1.0 ml·100 g body wt⁻¹·h⁻¹) through a cannula in the internal jugular vein. The temperature was maintained at 37.0 ± 0.5°C by means of a heating pad (Homeothermic Blanket Control Unit 50–7061; Harvard Apparatus) and monitored with a rectal probe thermometer. The rats were heparinized with 100 IU/kg heparin.

Surgical Preparation

The trachea was cannulated (polyethylene tubing, PE-240, Beckton-Dickinson) to allow spontaneous respiration. A carotid artery-jugular vein arteriovenous shunt was established as previously described (12). Arterial blood samples (25 μl) were taken by puncture of the silicone tubing in the shunt. Arterial blood pressure was monitored by briefly clamping the venous outlet. The patency of flow in the shunt was also monitored by recording pressure from the nonoccluded loop. Insulin, glucose, and anesthetic were administered intravenously by puncturing the loop on the venous side [infusion line PE-50 (Becton-Dickinson), with a cut 23-g needle at the delivery end]. The portal vein was cannulated with a 24-g intravenous catheter (Jelco; Johnson & Johnson Medical) after laparotomy. The rats were allowed to stabilize from the surgical intervention for 50 min before any procedures were carried out. Arterial blood samples were collected every 5 min after stabilization, and glucose concentrations were immediately analyzed by the oxidase method with a glucose analyzer (1500 YSI SPORT; Yellow Springs Instruments) until three successive stable glucose concentrations were obtained. The mean of these three values is to the baseline glucose level and was used as the euglycemic target for the rapid insulin sensitivity test (RIST).

RIST

The RIST is a modified euglycemic clamp that shows reproducibility for four consecutive tests performed in the same anesthetized animal (12) and is not affected by pentobarbital sodium anesthesia in the rat (8).

Insulin infusion was started using an infusion pump (Perfusor; B-Braun) to administer a bolus of 50 mU/kg iv over 5 min. To avoid hypoglycemia, the glucose infusion (d-glucose, 100 mg/ml iv) was started at a rate of 5 mg·kg⁻¹·min⁻¹, 1 min after onset of the insulin infusion. According to arterial glucose concentrations measured at 2-min intervals, the infusion rate of the glucose pump was readjusted to maintain euglycemia. When no further glucose infusion was required, usually within 35 min, the test period was concluded. The amount of glucose infused after insulin administration quantifies insulin sensitivity and is referred to as the RIST index (mg glucose/kg body wt; see Ref. 12).

HISS Action Quantification

Blockade of HISS release is achieved by interruption of the hepatic parasympathetic nerves by surgical denervation (34), atropine (31), or inhibition of hepatic NOS (25, 4). The HISS-dependent component of insulin action is calculated by subtracting the RIST index after blockade of HISS release from the RIST index of the control response, as previously reported (10). The HISS-independent component, or insulin action per se, is equal to the RIST index after HISS blockade.

Experimental Protocols

Effect of intraportal administration of the NO donor SIN-1 on insulin sensitivity, after blockade of hepatic muscarinic receptors. The RIST index was determined before and after blockade of HISS release with atropine (3 mg/kg iv over 5 min). The NO donor SIN-1 was administered intraportally at a dose of 5 mg/kg (n = 7) or 10 mg/kg (n = 6). After a basal glucose level was established, a new RIST was performed.

Effect of intraportal administration of the NO donor SIN-1 or ACh on insulin sensitivity, after blockade of hepatic NOS. The RIST index was determined before and 60 min after infusion of L-NAME (1 mg/kg iv, 5-min bolus), the time required to achieve maximal L-NAME effect after intraportal drug infusion (3). After the second RIST, SIN-1 (5 mg/kg, n = 5) or ACh (2.5 μg·kg⁻¹·min⁻¹, n = 7 and 5 μg·kg⁻¹·min⁻¹, n = 6) was infused intraportally, and the RIST was repeated.

Is GC involved in HISS action? Because the GC inhibitor, ODQ is dissolved in ethanol, we performed a control RIST followed by a RIST in the presence of 0.3 ml/kg 1% ethanol (iv, n = 6) to assess its influence on insulin sensitivity. In another set of experiments, ODQ was infused intraportally (n = 5) and intravenously (n = 7) to test GC influence in insulin sensitivity. The RIST index was determined before and after infusion of ODQ at a dose of 5 nmol/kg.

Drugs

Atropine sulfate, L-NAME, SIN-1, acetycholine chloride, ODQ, and d-glucose were purchased from Sigma-Aldrich, human insulin (HUMULIN, regular) was from Lilly, pentobarbital sodium (Eutasisil) was from Sanofi, and heparin was from B-Braun. All chemicals were dissolved in saline, except for ODQ, which was dissolved in 1% ethanol (Merck).

Data Analysis

Data were analyzed using repeated-measures ANOVA, followed by the Tukey-Kramer multiple-comparison test in each group. ODQ data were analyzed using an unpaired two-tailed t-test. The data are expressed as means ± SE throughout. Differences from control were accepted as statistically significant at P < 0.05.
RESULTS

Effect of Intraportal Administration of the NO Donor SIN-1 on Insulin Sensitivity, After Blockade of Hepatic Mucarincic Receptors

In the first set of animals, administration of atropine (3 mg/kg iv) reduced the RIST index from 215.6 ± 18.4 to 123.2 ± 7.4 mg glucose/kg, \( P < 0.001 \) (42.4 ± 2.7% inhibition, \( n = 7 \)). SIN-1 (5 mg/kg ipv) did not fully restore the insulin response (143.7 ± 20.2 mg glucose/kg), as shown in Fig. 1A.

In the second set of animals, administration of atropine (3 mg/kg iv) reduced the RIST index from 209.7 ± 21.3 mg glucose/kg, \( P < 0.01 \), corresponding to 43.3 ± 1.8% inhibition, \( n = 6 \). SIN-1 at a higher dose (10 mg/kg iv) fully restored the insulin response (288.3 ± 15.5 mg glucose/kg), as shown in Fig. 1B.

Effect of Intraportal Administration of the NO Donor SIN-1 or ACh on Insulin Sensitivity, After Blockade of Hepatic NOS

The control RIST index was 271.3 ± 37.6 mg glucose/kg. Intraportal administration of \( L \)-NAME (1 mg/kg) significantly reduced the RIST index to 152.2 ± 21.3 mg glucose/kg, \( P < 0.01 \), corresponding to 43.4 ± 2.1% inhibition. Intraportal administration of SIN-1 (5 mg/kg) restored insulin responsiveness (321.7 ± 44.7 mg glucose/kg, \( n = 5 \); Fig. 2).

In another set of animals, intraportal administration of \( L \)-NAME (1 mg/kg) reduced the RIST index from 257.1 ± 20.3 to 134.4 ± 8.9 mg glucose/kg, \( P < 0.001 \) (49.0 ± 3.6% inhibition). ACh (2.5 \( \mu \)g·kg\(^{-1} \)·min\(^{-1} \) iv) did not restore the RIST index to control values (137.2 ± 14.8 mg glucose/kg, \( P < 0.001 \) from control, \( n = 7 \)), as shown in Fig. 3A. A dose of 5 \( \mu \)g·kg\(^{-1} \)·min\(^{-1} \) ACh intraportally did not restore the insulin sensitivity after \( L \)-NAME administration as well, since the post-ACh RIST is 109.4 ± 11.9 mg glucose/kg (\( P < 0.001 \) from control, \( n = 6 \); Fig. 3B).

Is GC Involved in HISS Action?

After intraportal perfusion of 0.3 ml/kg of 1% ethanol, the RIST index was not altered significantly (from 209.7 ± 15.5 to 182.4 ± 15.0 mg glucose/kg).

Intraportal administration of ODQ (5 nmol/kg) reduced insulin sensitivity from 237.6 ± 18.6 to 111.7 ± 6.2 mg glucose/kg, corresponding to 51.0 ± 6.8% inhibition (\( n = 5 \)). Intravenous administration of ODQ (5 nmol/kg) led to a change from 198.9 ± 10.9 mg glucose/kg in control to 167.4 ± 10.9 mg glucose/kg after intravenous ODQ (\( n = 7 \)), corresponding to 15.6 ± 4.2% inhibition (\( P < 0.001 \), Fig. 4).

DISCUSSION

The purpose of this study was to clarify the role of ACh, NO, and the classical NO receptor, GC, in HISS action.
Our data show that ACh does not reverse HISS-dependent insulin resistance produced by blockade of NO synthesis in the liver. In contrast, HISS-dependent insulin resistance produced either by hepatic muscarinic receptor antagonism or by NOS blockade is reversed by NO donors. Our results suggest that NO donors are a powerful pharmacological tool to restore HISS action, both in cases of hepatic parasympathetic blockade and hepatic NOS antagonism. We have also observed that hepatic GC inhibition leads to insulin resistance. This suggests that HISS action is dependent on GC activity.

Methodological Considerations

HISS action was quantified using the RIST. Similar, although less precise, quantification can be attained using the arterial-venous glucose gradients across vascular beds (18, 33) or the insulin tolerance test (20, 34). The RIST was selected to quantify insulin sensitivity because it can be carried out four consecutive times in the same animal with high reproducibility, permitting paired experimental design (12). The RIST is effective in anesthetized or conscious animals and provides similar results independently of pentobarbital sodium anesthesia (8). The precision and accuracy of the RIST can be assessed by quantifying the deviation from the ideal euglycemic target.

A critical aspect of our study involves the use of ODQ to elucidate the role of GC in HISS action. We have previously reported that methylene blue (MB), a nonspecific GC inhibitor, also induced insulin resistance when administered intraportally in rats (2); however, MB has been described as being a weak inhibitor of GC that shows additional anticholinergic effects. ODQ is a much more potent and specific inhibitor of GC, being an important pharmacological tool in differentiating cGMP-dependent and -independent effects (6).

The dose of SIN-1 (10 mg/kg) needed to restore insulin sensitivity after muscarinic blockade with atropine was higher than the dose needed to reverse t-NAME-induced HISS inhibition (5 mg/kg). At present, we have no conclusive explanation for these data, but it seems possible that an atropine-induced increase in intracellular cAMP levels, resulting from blockade of inhibitory muscarinic input to the adenylate cyclase system (30), could stimulate cGMP-specific phosphodiesterases through cAMP/cGMP cross talk, leading to a decrease in basal cGMP levels (1). The hypothesis that atropine stimulates cGMP degradation might explain the need to use higher doses of SIN-1 to produce the amount of cGMP required to restore HISS action.

Importance of NOS in HISS Release

We observed that intraportal administration of the NO donor SIN-1 was able to reverse the insulin resistance induced by atropine. On the other hand, intraportal administration of ACh in a dose that reverses insulin resistance induced by hepatic parasympathetic denervation (2.5 μg·kg⁻¹·min⁻¹) and in a dose two times higher (5 μg·kg⁻¹·min⁻¹) was not effective in restoring HISS action after NOS antagonism. The fact that ACh cannot restore HISS action if NOS is blocked, whereas SIN-1 can, suggests that NOS is one of the effectors for the
hepatic muscarinic receptors in the HISS pathway. Therefore, we suggest that the permissive action of the hepatic parasympathetic nerves is to assure the release of endogenous NO in the liver required for HISS secretion in response to insulin.

The source of hepatic NO that controls the HISS mechanism is not clear. Recently, Porszasz et al. (19) proposed that the NO involved in HISS release is of sensory neural origin, based on the observation that sensory (capsaicin-sensitive) denervation of the anterior hepatic plexus (AHP) leads to insulin resistance, which they have interpreted is HISS-dependent insulin resistance. However, all of the experiments were performed in Wistar rats fasted for 24 h, which according to our results leads to full blockade of the HISS mechanism. The HISS-dependent and HISS-independent actions were not differentiated in those studies; thus, the deleterious effect that selective sensory denervation of the AHP has on insulin sensitivity is most likely HISS independent.

Interestingly, our results imply that HISS-dependent insulin resistance associated with hepatic neuropathy and NOS impairment can be overcome by administration of NO donors in rats. In contrast, muscarinic agonists are unlikely to be effective in restoring HISS-dependent insulin resistance secondary to defective hepatic NO production, although they may be effective in cases of parasympathetic impairment (32).

These results highlight that any pharmacological approach to reverse HISS-dependent insulin resistance has to focus on the specific defect leading to the resistant state. It is highly unlikely that the HISS-dependent insulin resistance reported in several animal models, including the chronic bile duct ligation model of liver disease (13), high-sucrose diet model of type 2 diabetes (21), obesity model (22), hypertension model (23), fetal alcohol exposure model (26), and aging model (17), is caused by a single defective step in the HISS pathway. Therefore, it is also highly unlikely that the HISS-dependent insulin resistance observed in these pathological models could be treated by the same therapeutic target. To choose the more suitable therapeutic approach, the defect in the HISS pathway needs to be identified.

**Involvement of GC in HISS Release**

Our data show that the GC inhibitor ODQ caused additional insulin resistance when administered intraperitoneally compared with intravenously. This indicates that GC in the liver, and not in the periphery, is involved in the control of insulin sensitivity. Sadri and Lautt (25) also observed that insulin resistance is strongly related to inhibition of NOS in the liver, and not in the peripheral tissues. Our results endorse the hypothesis that the HISS-dependent component of insulin action is directly related to NO production and GC activation in the liver, and not in skeletal muscle or the vasculature.

A major action of NO is the direct activation of the GC/cGMP system; however, GC is only one of the possible targets for NO produced in the liver. NO has other effects in vivo, namely reactions with DNA (27), thiol-containing proteins, and free thiols, to form S-nitrosothiols (29).

We have recently reported that hepatic GSH, together with NO, is determinant in allowing full HISS-dependent insulin action, probably through formation of nitrosylated intermediates (4). The nitrosylated derivative of GSH, S-nitrosoglutathione (GSNO), can act as an endogenous NO reservoir, since this compound is a stable molecule and a biologically active NO adduct (5). GSNO can trigger GC to stimulate cGMP synthesis (5), which suggests that GSNO may be an intermediate between NO synthesis and GC activation in the HISS pathway.

On the other hand, GSNO can also trigger GC-independent events by either S-nitrosylation or S-thiolation of other proteins (5). This process has been described recently as leading to modulation of enzyme activity and regulation of signal transduction pathways (24). Whether GSNO is a nitrosating/thiolating activator of key enzymes, or simply a stable NO pool that activates GC in a regulated manner allowing HISS secretion, remains to be clarified.

It has been proposed that methods to mimic or potentiate the hepatic parasympathetic permissive signal may be used to cause the release of HISS and thereby reverse HISS-dependent insulin resistance. The present data confirm that delivery of hepatic NO is also a potential pharmacological approach but also, and for the first time, suggest that hepatic GC is involved in HISS-dependent insulin action. Further studies are required to elucidate its interactions with NO and GSH and its role in HISS release.

**ACKNOWLEDGMENTS**

We greatly acknowledge P. F. Costa and A. I. Santos for helpful discussions.

**GRANTS**

This work was supported by the Portuguese Ministry of Science and Technology, Science and Technology Foundation (FCT) Grants POCI/SAU/14009/1998 and POCI/INE/42397/2001, and by the Portuguese Diabetes Association. M. P. Guarino was supported by FCT fellowship BD/4916/2001.

**DISCLOSURES**

W. W. Lautt is the President and Chief Scientific Officer of DiaMedica Inc., licensor of technology to treat HISS-dependent insulin resistance.

**REFERENCES**


