Acetylcholinesterase Antagonist Potentiated Insulin Action in Fed but Not Fasted State

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ABSTRACT

The glucose disposal effect of insulin is doubled in response to a meal. This meal-induced insulin sensitization results from insulin acting on the liver, in the presence of a permissive hepatic parasympathetic feeding signal and elevated hepatic glutathione (GSH), to release hepatic insulin-sensitizing substance (HISS), a hormone that acts selectively on skeletal muscle to stimulate insulin-mediated glucose uptake. Blockade of the parasympathetic feeding signal to the liver, either through surgical denervation or atropine-mediated antagonism of hepatic muscarinic receptors, eliminates the HISS response, resulting in HISS-dependent insulin resistance (HDIR) and decreasing the response to insulin by approximately 55% in the fed state. Insulin action in Sprague-Dawley rats, as determined with a rapidly sampled, transient euglycemic clamp in response to insulin (50 mU/kg), is decreased in a dose-dependent manner by atropine. In this study, we have used the ED75 atropine-induced model of HDIR. After a submaximal dose of atropine, potentiation of the remaining parasympathetic effect with the acetylcholinesterase antagonist neostigmine significantly restored postprandial insulin sensitization in a dose-dependent manner with peak effect at 0.1 μg/kg/min. Neostigmine reversed the insulin resistance induced by partial fasting and partial muscarinic inhibition (hepatic GSH levels are at fed levels), but not that induced by surgical hepatic denervation (GSH normal, no nerve signal) or 24-h fasting (low GSH). Neostigmine potentiated the response to insulin by neostigmine occurred in normal, fed rats. The data suggest the use of either direct or indirectly acting cholinergic agonists for the treatment of impaired postprandial insulin sensitization.

The glucose disposal effect of administered insulin is approximately doubled in response to feeding (Lautt et al., 2001). In the immediate postmeal state insulin causes the release of a factor from the liver. This putative hormone has been termed hepatic insulin-sensitizing substance (HISS) (Lautt, 1999). HISS action is proposed to selectively account for the large increase in postprandial glucose sequestration attributable to HISS production and release by the liver. Extensive in vivo studies have revealed much about the physiological and pharmacological characteristics of the putative hormone without revealing its chemical identity (Lautt, 2004 and reviewed in Lautt and Ming, 2010). HISS action in vivo is readily quantified.

HISS is not released in the fasted state, but becomes maximally releasable within 90 min of eating, resulting in MIS after a meal (Sadri et al., 2006). The release of HISS from the liver depends on a permissive parasympathetic nerve signal and hepatic glutathione (GSH) levels. These two essential “feeding signals” can be completely blocked by various means, including surgical or pharmacological interruption of the hepatic parasympathetic nerves (Xie et al., 1993; Xie and Lautt, 1995), inhibition of the production of hepatic nitric oxide (Sadri and Lautt, 1999), hepatic cyclooxygenase inhibition (Sadri and Lautt, 2000), and the depletion of hepatic GSH (Guarino et al., 2003, 2004). The blockade of either of the feeding signals leads to blockade of HISS action and a lack of MIS. In the absence of MIS there is a development of postprandial hyperglycemia, hyperinsulinemia, hyperlipidemia, and increased reactive oxidative stress with the concomitant cardiometabolic risk associated with these conditions (Lautt, 2007; Lautt et al., 2008).

In the fed state, HISS-dependent insulin action accounts for approximately 55% of insulin’s glucose disposal effect,
whereas the remaining 45% is accounted for by the direct action of insulin and is termed the HISS-independent component of insulin action. Blockade of HISS release is said to produce HISS-dependent insulin resistance (HDIR) and may account for the majority of the insulin resistance seen in type 2 diabetes, hypertension, chronic liver disease, and obesity. The process of normal aging recently has been shown to result in the development of HDIR, the progression of which was accelerated by dietary supplementation with sucrose and delayed by the provision of an antioxidant cocktail (Lautt et al., 2008; Ming et al., 2009).

Importantly, in some conditions, such as disease or aging, there is impairment in the release of acetylcholine by the parasympathetic nerves (Terry and Buccafusco, 2003). During normal nervous system function, acetylcholine is rapidly degraded by acetylcholinesterase in the synaptic cleft. However, where acetylcholine release is below normal levels it may be desirable to enhance the effectiveness of the remaining neurotransmitter that is released by prolonging its duration in the synaptic cleft. This approach is commonly used for the treatment of the deficit in somatic nervous system signaling in myasthenia gravis through blockade of acetylcholine degradation at the site of nicotinic receptors in muscle. Increasing the time that acetylcholine endures in the synaptic cleft, through antagonism of acetylcholinesterase enzymes, allows for greater interaction between the ligand and its receptors on the postsynaptic site and amplifies any signal. Here, we attempt to address whether a similar strategy can be used to potentiate the parasympathetic nervous system signal in the liver.

Previously, in both feline models (Xie and Lautt, 1995) and rodent models (Takayama et al., 2000), the glucose disposal response to exogenously administered insulin was shown to be progressively decreased in a dose-dependent manner by atropine, to a maximum of 55% inhibition. The equivalent phenomenon was demonstrated in humans with a 56.5% reduction in postprandial insulin sensitivity achieved by a submaximal dose of atropine (Patarra˜o et al., 2008). In this study, the induction of experimental insulin resistance was achieved pharmacologically through the administration of a submaximal (ED75) dose of the muscarinic antagonist atropine. The HDIR was then reversed through the use of intraportal venous (i.p.v.) administration of the acetylcholinesterase inhibitor neostigmine, thereby restoring normal postprandial insulin sensitivity and MIS. Neostigmine potentiated HISS release in 8-h fasted rats, but failed to do so in 24-h fasted rats or fed rats that had parasympathetic nerves sectioned. The data suggest the use of indirect-acting cholinergic agonists for the treatment of impaired postprandial insulin sensitivity such as in the prediabetic condition.

Materials and Methods

Animals were treated according to the guidelines of the Canadian Council on Animal Care, and the Protocol Management and Review Committees at the University of Manitoba approved all protocols. Male Sprague-Dawley rats (300 ± 50 g) were housed in climate-controlled conditions and fed ad libitum with standard laboratory rat chow and free access to tap water.

Surgical Preparation. Before acute experimentation, animals were fasted for 8 h, and then refed for 2 h (except as otherwise noted) before being anesthetized with an injection of pentobarbital sodium (65 mg/kg i.p., Somnotol; Biomedia-MTC Animal Health Inc., Cambridge, Canada). The in vivo surgical preparation has been detailed elsewhere (Lautt et al., 1998) and includes femoral cannulation with an arterial-venous shunt, tracheotomy to allow spontaneous respiration, jugular venous cannulation for administration of supplemental anesthesia and d-glucose-saline, and a laparotomy with portal venous puncture using a 24G intravenous catheter (Optiva; Smiths Medical ASD, Inc., Southington, CT) for delivery of neostigmine at selected doses. Body temperature was maintained at 37.5 ± 0.5°C throughout all experiments by using a rectal probe and a heated thermal surgical table with a closed-loop feedback system. The physiological parameters of heart rate and blood pressure were monitored and recorded, and the infusion pumps were controlled with a computerized semiautomated system. After the surgical preparation, animals were allowed to stabilize for 30 min before initiation of sampling.

Rapid Insulin Sensitivity Test. The rapid insulin sensitivity test (RIST) technology has previously been fully described (Lautt et al., 1998; reviewed in Lautt, 2003) and is a transient, rapidly sampled, hyperinsulinemic, euglycemic clamp in response to a standardized bolus of insulin. In brief, baseline blood glucose levels were determined with a YSI glucose analyzer (using the glucose oxidase method) (Yellow Springs Instruments, Yellow Springs, OH), by means of samples taken at 5-min intervals and continuing until three successive stable determinations were made. The mean of these three samples was taken as the euglycemic baseline to be maintained during the RIST. Insulin infusion was commenced by using an insulin pump to administer 50 mU/kg, in a 0.5-ml bolus, over 5 min into the venous side of the arterial-venous shunt. Arterial glucose levels were sampled at 2-min intervals with intravenous glucose infusion rates adjusted to maintain euglycemia. The RIST index is the area under the glucose infusion curve, represents the total amount of glucose infused during the clamp, and is standardized to body weight and expressed as mg glucose/kg.

ED$_{50}$ Atropine Model of HDIR. The ED$_{50}$ atropine model of HDIR requires the development of reversible insulin resistance through the administration of a submaximal inhibitory dose of the muscarinic antagonist atropine. In this study, an initial RIST was performed to measure the insulin sensitivity in the control fed state. Atropine was then administered at various doses over the range of 1 × 10$^{-7}$ to 1 × 10$^{-5}$ mg/kg, and a second RIST was conducted. A full dose-response curve was plotted as the dose of atropine versus the proportion of the maximal blocking effect of atropine produced by the given dose. Data were transformed by using nonlinear curve fitting to determine the ED$_{50}$ dose.

Dose Response Testing of Neostigmine. Insulin sensitivity was determined in the control fed state. After the control RIST1, the ED$_{50}$ atropine dose (5.01 × 10$^{-5}$ mg/kg i.v.) was administered, and a second RIST2 was performed. The acetylcholinesterase antagonist neostigmine was then administered over a dose range of 0.010 to 2 µg/kg/min at a continuous infusion rate (0.025 ml/min) into the portal vein. Upon glycemic stabilization, a neostigmine RIST3 was carried out. RIST index values were compared within the same animal to determine the capacity of the neostigmine dose to reverse the atropine-induced HDIR. The calculation of percentage of reversal is derived by (neostigmine RIST3 − postatropine RIST2)/control RIST1 × 100%. The determination of percentage of inhibition is (control RIST1 − postatropine RIST2)/control RIST1 × 100%, and percentage of potentiation is (neostigmine RIST3 − postatropine RIST2)/postatropine RIST2 × 100%. A dose-response curve for percentage of reversal was generated, and the optimal dose of neostigmine was determined.

Neostigmine Effect on Normal Fed Rats. Control levels of insulin sensitivity were determined in the fed state after an 8-h fast and 2-h refeed ($n = 4$). Upon completion of the control RIST, neostigmine was infused, at the optimal dose determined from the dose-response curve, and a second RIST was carried out. This was followed by intravenous administration of atropine at a dose of 1 mg/kg that had been previously determined to generate maximal inhibition
of HISS release for a sufficient duration of action to carry out the RIST in the rat (Takayama et al., 2000), and a third RIST was conducted. A parallel series of experiments was conducted with a control RIST, neostigmine RIST, and atropine RIST in normal animals but where a high dose (1 μg/kg/min) of neostigmine was administered instead of the optimal dose (0.1 μg/kg/min) as indicated above. A further series of experiments was then conducted identically, but with the high dose of neostigmine administered in conjunction with phenolamine (400 μg i.p.v.) to determine the effect of α-adrenergic blockade on neostigmine actions.

Fasting-Induced Models of HDIR. Two series of experiments were conducted to assess the effect of fasting duration on insulin sensitivity and measure the effect of neostigmine activity. In the 8-h fasting-induced HDIR model (n = 4), animals were fasted for 8 h, refed for 2 h, and then fasted for 8 h before the start of acute testing. This fasting refeding procedure was used to standardize the volume and timing of the final meal. In the 24-h fasting-induced HDIR model (n = 4), animals were fasted for 8 h, refed for 2 h, and then fasted for 24 h. In both fasting-induced models of HDIR, the control level of insulin sensitivity was determined by using RIST methodology, and then neostigmine was administered at the optimal dose and a second RIST was performed. After this, a full blocking dose of atropine was administered (1 mg/kg i.v.), and a final RIST test was carried out.

Surgically Induced Model of HDIR. The effect of neostigmine on surgical denervation-induced HDIR was studied as per the atropine model described above with the following differences. During the initial surgical preparation the laparotomy was performed without portal puncture. Animals (n = 4) were then stabilized, and a control RIST was performed. After the control RIST, a second surgical intervention was carried out where the anterior hepatic plexus was isolated and transected at the junction of the celiac and common hepatic arteries, and then the portal vein was cannulated. Animals were allowed to restabilize, and then a postdenervation RIST was performed. After this, neostigmine was infused at the optimal dose, and a third RIST was carried out.

Drugs. Human insulin (Humulin R) was obtained from Eli Lilly & Co. (Toronto, Canada). D-Glucose, neostigmine, phenolamine, and atropine were purchased from Sigma-Aldrich (St. Louis, MO). All drugs were freshly made for each experiment and dissolved in physiological saline.

Glutathione Measurement. At the conclusion of the acute experiment, liver biopsy samples were rapidly excised from the left lateral lobe by using a cork borer (8-mm diameter). The top and bottom 2 mm of tissue were trimmed off, and the remaining core sample was immediately frozen on dry ice and then stored at −80°C until analysis. Total hepatic GSH was determined by using quantitative colorimetric analysis with the Bioxytech GSH-420 assay kit (Oxis Health Products, Inc., Portland, OR). In brief, livers were homogenized in phosphate-buffered (pH 7.8), 7.5% ice-cold trichloroacetic acid solution. Homogenates were centrifuged at 3000g for 10 min at 4°C, and the supernatant was used for the assay. Total GSH content was measured at 420 nm by using an Ultraspec 4000 UV/visible spectrophotometer (GE Healthcare, Little Chalfont, Buckinghamshire, UK) using a method based on the formation of chromophoric thione. In this procedure, absorbance measured at 420 nm is directly proportional to the total GSH concentration, is standardized to a known GSH sample, and is corrected to the total mass of liver tissue sampled.

Statistical Analysis. Results are expressed throughout as mean ± standard error. Statistical significance was assessed by repeated measures analysis of variance (ANOVA) using Tukey’s posttest with significance accepted at p < 0.05 and lack of significance (p > 0.05) indicated.

Results

Atropine Inhibits Insulin Sensitivity in a Dose-Dependent Manner. Atropine dose-dependent inhibition of insulin sensitivity has been reported in the rat (Takayama et al., 2000) and the cat (Xie and Lautt, 1995). Some degree of neural activation must remain for the anticholinesterase compounds to be effective. To establish an appropriate dose of atropine that resulted in reversible insulin resistance, we determined a dose-response curve for intravenous atropine over a dose range of 1 × 10⁻⁷ to 1 × 10⁰ mg/kg. The percentage of inhibition of insulin sensitivity by atropine was determined by comparing the RIST index before and after atropine in the same (fed) animal. The percentage of inhibition by atropine was then standardized, and the data were expressed at each dose as a proportion of the maximal blocking effect of atropine with the full dose-response curve shown. (Fig. 1). Nonlinear regression curve fitting determined the ED₇⁵ dose of atropine to be 5.01 × 10⁻⁶ mg/kg.

ED₇₅ Atropine Model of HDIR. Administration of a submaximal inhibitory dose of atropine (ED₇₅ 5.01 × 10⁻⁶ mg/kg) produced an insulin resistance (HDIR) that was reversible by the administration of intraportal neostigmine. Pooled data (n = 25) demonstrated a mean control RIST index of 194.1 ± 5.4 mg/kg. Intravenously administered atropine (5.01 × 10⁻⁶ mg/kg) resulted in a statistically significant (p < 0.001) inhibition from control insulin sensitivity (45.4 ± 1.5%) to a mean postatropine RIST index value of 105.3 ± 3.4 mg/kg. Pooled results from experiments examining the full blockade by atropine (1 mg/kg) from the control, fed level of insulin sensitivity showed an inhibition of 58.0 ± 3.3% (n = 13). A predicted 75% inhibition of the insulin sensitivity from maximal blockade of 58% would have been 43.5% inhibition. This corresponded closely to the observed inhibition of 45.4% by the ED₇₅ dose in these experiments.

Several doses of neostigmine were tested for reversal of ED₇₅ atropine inhibition (Fig. 2, B and C), then a full dose response...
range was generated (Fig. 3), and the peak reversal dose was determined to be 0.1 μg/kg/min.

In the group of animals tested at the peak reversal dose of neostigmine (0.1 μg/kg/min; n = 3), the initial dynamic insulin sensitivity was determined under fed conditions, and the control RIST index was 178.7 ± 4.8 mg/kg. The ED$_{75}$ atropine dose resulted in a significant (p < 0.001) inhibition (47.0 ± 5.5%) of the RIST index values to 94.3 ± 7.6 mg/kg. The atropine-inhibited RIST was significantly potentiated (68.5 ± 7.0%, p < 0.01) by neostigmine at this dose to a level of 157.8 ± 6.7 mg/kg that was not significantly different from control (N.S., p > 0.05) and represented a reversal of 77.9 ± 9.0% (Fig. 2A).

The full dose-response curve for intraportal neostigmine demonstrated a bell-shaped curve with the highest doses tested resulting in a less effective reversal of the HDIR (Fig. 3).

**Effect of Neostigmine in the Normal Fed Animal with Intact Nerves.** The previous study (Fig. 2) suggested that neostigmine would be effective in physiological conditions where hepatic parasympathetic activity is decreased or impaired. Neostigmine was administered to normal control animals to evaluate the effect of the anticholinesterase compound on fully functioning parasympathetic nerves. In fed animals with intact hepatic nerves (n = 4), the infusion of 0.1 μg/kg/min neostigmine (RIST index 162.3 ± 4.6) had no significant effect on insulin sensitivity from control fed levels (175 ± 1.8 mg/kg). The full atropine inhibitory dose (1 mg/kg) produced a significant inhibition (RIST index 72.5 ± 3.9) of 58.8 ± 3.3% (p < 0.001) from control levels (Fig. 4A). Neither neostigmine nor atropine administration affected the basal parameters of arterial blood pressure and basal glycemia as determined from values measured immediately before the control, the postneostigmine, and the postatropine RISTs (arterial pressure: 97.2 ± 5.1, 94.6 ± 2.8, and 90.0 ± 2.2 mmHg).

![Fig. 2. Administration of an ED$_{75}$ dose of atropine resulted in a significant reduction in insulin sensitivity from control that was reversible by intraportal neostigmine infusion at various doses. A, inhibition by ED$_{75}$ atropine 47.0 ± 5.5%. Potentiation by neostigmine (0.1 μg/kg/min) was 68.5 ± 7.0%, which represented a reversal of 77.9 ± 9.0% (n = 3). B, ED$_{75}$ atropine inhibition 46.2 ± 4.9%. Potentiation by neostigmine (1.0 μg/kg/min) was 45.1 ± 5.9%, with a reversal of 51.0 ± 6.2% (n = 5). C, ED$_{75}$ atropine inhibition 44.5 ± 2.2%. Potentiation by neostigmine (2.0 μg/kg/min) was 30.1 ± 2.9, and the reversal was 38.9 ± 4.7% (n = 6), showing that the lower dose was more effective. Based on these observations, a dose-response curve was determined (Fig. 3).](image-url)
Hg, respectively, N.S. (basal glycemia: 106.6 ± 3.2, 106.0 ± 2.2, and 100.9 ± 2.9 mg/dL, respectively, N.S.).

These data indicated that the optimal dose of neostigmine (0.1 μg/kg/min) did not have a stimulatory effect on insulin sensitivity in normal fed rats. To elucidate the mechanism for the reduced effect of neostigmine observed at higher doses (Fig. 3) a comparative study was conducted with a neostigmine dose that was 10 times higher (1.0 μg/kg/min). Similar to the dose-response curve experiments, the control fed RIST index was significantly decreased (p < 0.001) by the high dose of neostigmine to a level that was further significantly reduced (p < 0.001) by the full blocking dose of atropine (150.4 ± 5.4 mg/kg; n = 4). Neither neostigmine nor atropine affected the basal parameters of arterial blood pressure and basal glycemia as measured immediately before the control, the postneostigmine, and the postatropine RISTs (data not shown).

**Sympathetic Nerve Blockade Inhibits High-Dose Neostigmine-Induced HDIR.** To test the hypothesis that the decreased insulin sensitivity that was observed at higher doses of neostigmine was a consequence of sympathetic nerve activation, phentolamine, a nonselective α-adrenergic antagonist, was administered in conjunction with neostigmine. If the high dose of neostigmine resulted in systemic increase in acetylcholine that was affecting sympathetic nerve activity, then coadministration of phentolamine with the neostigmine would potentially reverse the sympathetic activation and reduce the observed HDIR. In this series of experiments (n = 4), a control RIST index was determined in fed animals (172.1 ± 1.1 mg/kg). Administration of the α-blocker phentolamine (400 μg/kg i.p.v.) was immediately followed by neostigmine (1.0 μg/kg/min i.p.v.), and upon attainment of glycemic stability a postphentolamine and neostigmine RIST index was determined (167.7 ± 7.7 mg/kg) and it was not significantly different from control levels of insulin sensitivity. Subsequently, a full inhibitory dose of atropine (1 mg/kg) resulted in greatly reduced insulin sensitivity (66.8 ± 5.5 mg/kg).
was not significantly different before the control, postphentolamine/neostigmine, and postatropine RISTs (91.5 ± 5.6, 87.6 ± 2.2, and 80.1 ± 5.1 mm Hg, respectively, N.S., n = 4). The basal glycemia was not significantly different between the control and postphentolamine/neostigmine RISTs (110.9 ± 4.3 versus 109.4 ± 4.8 mg/dL) but was significantly reduced after atropine administration (95.4 ± 5.3 mg/dL, p < 0.04, n = 4).

**Effect of Neostigmine in Denervation and Fasting-Induced Models of HDIR.** Using the dose of neostigmine (0.1 μg/kg/min) that produced the maximal reversal of ED75 atropine-induced HDIR, three other mechanisms of experimentally induced insulin resistance were examined: surgical hepatic denervation, 8-h partial fast, and 24-h fast (n = 4, all groups).

In comparison with the fully fed state, in the 8-h fasted condition there was a reduction in insulin sensitivity. The 8-h fasted RIST index (144.1 ± 0.6 mg/kg) was further inhibited significantly by a full-blocking dose (1 mg/kg) of atropine (81.1 ± 6.7 mg/kg). In 24-h fasted animals the insulin sensitivity (86.96 ± 4.01 mg/kg) was minimally reduced by the administration of a full atropine blockade to 75.1 ± 1.7 mg/kg. The steady progressive decrease in the insulin sensitivity observed with increasing duration of fasting represents a reduction in the HISS-dependent component of insulin sensitivity with postatropine insulin sensitivity (the HISS-independent component of insulin action) remaining relatively constant (Fig. 5).

The 8-h partial fasting-induced HDIR was significantly potentiated by the optimal dose of neostigmine (0.1 μg/kg/min) by 16.5 ± 4.4%. This was in contrast to the lack of potentiation by neostigmine of the 24-h fasting-induced HDIR (−0.7 ± 2.6%) and the surgical denervation-induced model (−4.9 ± 5.0%) (Fig. 6). In the fed denervation model, the control RIST index (175.5 ± 4.7 mg/kg) was significantly inhibited by 53.4 ± 1.4% in the postdenervation state (82.0 ± 1.8 mg/kg) and was not reversed by the administration of neostigmine (78.0 ± 5.4 mg/kg).

**Hepatic Glutathione Levels.** The results of the analysis of the liver biopsy for total hepatic glutathione content were pooled for all groups that were 8-h fasted and 2-h refed (n = 27) and were compared with the groups of animals that were 8-h fasted (n = 4) and 24-h fasted (n = 4). By the 8-h fasting time point there was no significant change in hepatic GSH (4.74 ± 0.26 μmol/g tissue) from control fed levels (4.75 ± 0.11). However, the level of hepatic GSH was significantly (p < 0.05, ANOVA) lower than control in the 24-h fasted state (3.83 ± 0.18 μmol/g tissue).
animals (3.83 ± 0.18 μmol/g tissue) (Fig. 7). GSH in the fed denervated group was 4.80 ± 0.35, similar to the fed group with nerves intact.

Discussion

Meal-Induced Insulin Sensitization. MIS is a consequence of HISS release that occurs in response to insulin after a meal. HISS action is decreased with increasing duration of fasting, under the regulation of two progressively declining feeding signals: the parasympathetic nervous signal and the hepatic glutathione signal (Guarino et al., 2003; Sadrí et al., 2006). For a meal to result in MIS, both feeding signals must be present.

The ability of a direct acting muscarinic agonist to mimic the parasympathetic nerve signal has been reported (Lautt et al., 2001). However, it was unclear whether indirect acting cholinergic agonists, which act by blocking the breakdown of acetylcholine, are capable of potentiating the cholinergic action of the parasympathetic nerves in the liver. In this study, the role of the parasympathetic nervous system in the development of HISS–HDIR was examined by using the acetylcholinesterase antagonist neostigmine and the muscarinic receptor antagonist atropine as pharmacological tools to manipulate the feeding signals with a view to developing novel therapeutic agents for the treatment of insulin resistance.

The ED\textsubscript{75} Atropine Model. To test the ability of neostigmine to potentiate the hepatic parasympathetic “feeding signal” we used atropine to produce a partial blockade of hepatic muscarinic receptors and determined the capacity of neostigmine to restore function. An acute model of insulin resistance was established in the fed, anesthetized rodent by using an ED\textsubscript{75} dose of 5.01 × 10\textsuperscript{-6} mg/kg atropine to generate 75% of the maximal pharmacological blockade of the HISS-dependent component of insulin sensitivity as determined by the RIST. The RIST is a methodology (Lautt et al., 1998) for measuring dynamic insulin action and has the key strength of being highly reproducible and repeatable in the same experiment multiple times. Studies using the RIST in rats with a full inhibitory dose of atropine (1 mg/kg) showed, over a wide range of insulin concentrations, that the HISS-dependent component of insulin sensitivity was 55.5 ± 3.5% of the total insulin action (Lautt et al., 2001). Based on a maximal inhibition of 58.0 ± 3.3% reported here (n = 13), 75% of this level would be approximately 43.5% inhibition of insulin action. This value corresponded well with the observed mean inhibition by the ED\textsubscript{75} dose of atropine (45.4 ± 1.5%, n = 25) tested in this study.

Potentiation of the remaining parasympathetic neurogenic feeding signal was achieved by administration of neostigmine. The capacity to reverse the ED\textsubscript{75} inhibition was demonstrated over a range of doses and shown to be maximally induced with an infused neostigmine dose of 0.1 μg/kg/min i.p.v. (Fig. 3). The decreased effectiveness of neostigmine that was observed at the highest doses tested (Fig. 2), the so-called “bell shape” of the curve (Fig. 3), may be caused by nonspecific, acetylcholine-mediated adrenergic stimulation, possibly as a result of autonomic ganglionic cholinergic action. This sympathetic nervous system signal is postulated to result in a physiological antagonism of the permissive parasympathetic signal for HISS release, leading to a sympathetic-induced, HISS-dependent reduction in peripheral insulin sensitivity. At the optimal dose of neostigmine there was no adverse effect on insulin sensitivity in the normal fed animal; however, at higher than optimal doses neostigmine inhibited the postprandial RIST index. Support for the hypothesis that at higher doses of neostigmine there was a cholinergic activation of both parasympathetic and sympathetic neural systems was provided by the use of the α-adrenergic blocker phentolamine. Pretreatment with phentolamine resulted in a preservation of insulin sensitivity in the innervated animal under treatment with high doses of neostigmine (Fig. 4). This series is consistent with the hypothesis that high doses of neostigmine resulted in sympathetic activation and resultant HDIR. The sympathetic activation appears to specifically affect the HISS-dependent component, because the postatropine insulin sensitivity (the HISS-independent component) was not significantly different between groups (Fig. 4).

The Two Essential Feeding Signals. Intraportal administration of acetylcholine was previously shown to reverse insulin resistance produced by hepatic surgical denervation (Xie and Lautt, 1996). If all of the effect of neostigmine is through the potentiation of the hepatic parasympathetic nerve signal, then denervation of the liver should eliminate the release of acetylcholine and the effect of neostigmine. Neostigmine failed to reverse the HDIR resulting from anterior hepatic plexus surgical denervation. In the absence of a functioning hepatic parasympathetic nerve bundle there is no delivery of acetylcholine to the muscarinic receptors in the liver and, even though the animal is in the fed state, there is no capacity for antagonism of the cholinesterase enzyme to potentiate the feeding signal. The permissive nature of the parasympathetic signal is demonstrated by the observation that continuous infusion of neostigmine did not significantly alter glucose levels but insulin, administered against this signal, demonstrated potentiated action. It also appears that the permissive signal cannot result in insulin sensitization beyond the level seen in a normal, healthy postprandial state, because neostigmine had no sensitizing effect in that condition.

The role of the second feeding signal for HISS release, the hepatic glutathione levels, has been previously demonstrated (Guarino et al., 2003). Meal-induced increase in GSH has been shown (Tateishi et al., 1997) and the depletion of hepatic glutathione levels with the γ-glutamylcysteine synthase blocker L-buthionine-[S,R]-sulfoximine was linked with the development of impaired glucose tolerance (Khamaisi et al., 2000). The insulin resistance that was generated by in vivo administration of L-buthionine-[S,R]-sulfoximine to reduce hepatic GSH was attributed to a HISS mechanism and shown to have no effect on the direct actions of insulin on skeletal muscle in vitro (Guarino et al., 2003).

Here, we examined the effect of two fasting durations (8 and 24 h) in the rat and showed that there was a progressive decrease in HISS action from the control state to the 24-h fasted condition (Fig. 5). Administration of neostigmine (0.1 μg/kg/min) in the 8-h partially fasted condition was able to reverse the HDIR, potentiating the insulin sensitivity by 16.5 ± 4.4% to a level similar to the fed state. The 24-h fasting HDIR, however, was not reversible by neostigmine. By 8 h of fasting there was not yet a reduction in measured GSH, whereas by 24 h there was a significant reduction of
GSH to 3.83 ± 0.18 μmol/g from control fed levels of 4.74 ± 0.26 μmol/g (Fig. 7).

This study sheds light on the timing of the deterioration of the two proposed permissive feeding signals that dictate the degree of HISS release and corresponding peripheral insulin sensitivity. By 8 h of fasting in the rat, the total hepatic GSH feeding signal has not yet begun to deteriorate, yet there is a reduction of insulin sensitivity via a HISS-based mechanism, which is reversible by using intraportal neostigmine. This would suggest that the hepatic parasympathetic feeding signal is the first component of the HISS release pathway to decline with fasting. By 24 h of fasting there is a significant reduction in the total hepatic GSH and a corresponding reduction in the whole body insulin sensitivity that is not further affected by atropine administration and is not reversible by intraportal neostigmine. This indicates that by the 24-h time point both feeding signals have deteriorated and the HDIR is maximal. Provision of only one of the feeding signals cannot restore HISS release (unpublished observation).

Effectiveness of neostigmine in diabetic models has not been evaluated. It is postulated that acetylcholinesterase inhibitors may be beneficial therapeutic agents for conditions of insulin resistance that may be related to HISS-based mechanisms, such as in type 2 diabetes (Lautt et al., 2008), whereby there is a reduction in the parasympathetic nervous feeding signal. The capacity of neostigmine to potentiate insulin sensitivity in the 75% atropine blockade model and reverse partial-fasting-induced HDIR is consistent with the HISS hypothesis. The lack of effect in the denervated liver and after a 24-h fast shows that the effect of neostigmine depended on functional hepatic parasympathetic nerves. The lack of effect of neostigmine in the presence of full parasympathetic action in the healthy fed state suggests that drug-induced hypoglycemia will not occur. Acetylcholinesterase inhibitors may be useful to restore HISS release and, therefore, MIR in conditions such as aging where there is a progressive deterioration of parasympathetic nerve function (Terry and Buccafusco, 2003) and a reduction in HISS release (Ribeiro et al., 2008; Ming, et al., 2009; Lautt and Ming, 2010).

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