Role of the autonomic nervous system in the development of hyperinsulinemia by high-carbohydrate formula feeding to neonatal rats

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Mitrani P, Srinivasan M, Dodds C, Patel MS. Role of the autonomic nervous system in the development of hyperinsulinemia by high-carbohydrate formula feeding to neonatal rats. Am J Physiol Endocrinol Metab 292: E1069–E1078, 2007. First published December 12, 2006; doi:10.1152/ajpendo.00477.2006.—An early dietary intervention in the form of a high-carbohydrate (HC) milk formula in neonatal rat pups results in immediate onset of hyperinsulinemia. While increased insulin secretion in HC rats has been shown to be related to hypersensitivity to glucose, the immediate onset of hyperinsulinemia and its persistence throughout the suckling period suggest involvement of multiple systems that enhance insulin secretion in response to increased demand. Evidence presented here in 12-day-old HC rats indicates that altered activity of the autonomic nervous system contributes to enhanced insulin secretory responses to glucose stimulation through increased parasympathetic and decreased sympathetic signaling. Both in vivo and in vitro studies have shown that HC rats secrete significantly higher levels of insulin in response to glucose in the presence of acetylcholine, a cholinergic agonist, while sensitivity to inhibition of insulin secretion by oxytazemoline, an α2-adrenergic receptor (α2-AR) agonist, was reduced. In addition, HC rats showed increased sensitivity to blockade of cholinergic-induced insulin secretion by the muscarinic type 3 receptor (M3R) antagonist 4-diphenylacetoxy-N-methyl-piperidine methobromide, as well as increased potentiation of glucose-stimulated insulin secretion by treatment with yohimbine. Increases in islets levels of M3R, phospholipase C-β1, and protein kinase Cα mRNAs, as well as decreased α2-AR mRNA, in 12-day-old HC rats provide a mechanistic connection to the changes in insulin secretion seen in HC rats. In conclusion, altered autonomic regulation of insulin secretion, due to the HC nutritional intervention, contributes to the development of hyperinsulinemia in 12-day-old HC rats.

high-carbohydrate milk formula; parasympathetic nervous system; sympathetic nervous system

RECENT EVIDENCE SUGGESTS that changes in the quality of nutrition during critical periods of early development (fetal and neonatal) may play a decisive role in the metabolic programming of early adaptive responses into adulthood which result in metabolic disease (6). Metabolic programming is the phenomenon in which a stimulus or insult that occurs during a critical period of organogenesis in early life results in permanent alterations in the structure and function of affected organs and increased susceptibility to adult onset of diseases (6). The late fetal and early postnatal periods of rat development constitute a period during which the ontogeny of the endocrine pancreas is vulnerable to nutritional perturbations that result in permanent structural and functional adaptations (9).

Studies from our laboratory found that an early high-carbohydrate (HC) dietary intervention during the immediate postnatal period (days 4-24) in rats results in the immediate onset of hyperinsulinemia and its persistence throughout adulthood, despite the removal of the HC nutritional stimulus at weaning (19, 20). HC rats display an enhanced insulin secretory response to glucose stimulation, in addition to other cellular, molecular, and biochemical changes that support a state of chronic insulin hypersecretion (20, 34, 38). While many components have been discovered that contribute to altered pancreatic function in HC rats, the exact mechanisms responsible for the immediate onset of hyperinsulinemia and its persistence into adulthood are still incompletely understood. The fact that glucose is the primary energy source for the central nervous system (CNS) demands that blood glucose levels be tightly regulated within a narrow range and, thus, a complex system of central and peripheral mechanisms is involved in the regulation of glucose homeostasis (23). Peripherally, circulating glucose levels directly control the secretion of insulin and glucagon, while the CNS is involved in central control of insulin and glucagon secretion through the autonomic nervous system (ANS) (7, 22, 23, 39). Therefore, the ANS, which undergoes substantial postnatal development that coincides with the HC dietary intervention (41), may play an important role in the programming of hyperinsulinemia in HC rats.

It is well-established that pancreatic islets are extensively innervated by vagal cholinergic nerves and sympathetic fibers which are involved in nutrient-mediated regulation of insulin secretion (31). The parasympathetic nervous system (PNS) innervating pancreatic islets release acetylcholine (ACh) in response to rising glucose levels during the absorptive phase of feeding, as well as a preabsorptive cephalic phase of insulin secretion (18). ACh stimulation of β-cells is known to occur primarily through activation of muscarinic type 3 receptors (M3R), resulting in enhanced glucose-stimulated insulin secretion (GSIS) (4, 23, 27) (Fig. 1). The sympathetic nervous system (SNS) activity is mediated through NE and epinephrine binding to adrenergic receptors located on target tissues. SNS regulation of insulin secretion is mediated by NE and epinephrine binding to the α2-adrenergic receptor (α2-AR) (29) which activates G protein-mediated inhibition of adenylate cyclase, resulting in reduced insulin secretion (13, 23) (Fig. 1). While these two systems typically act in opposing fashions, existing evidence indicates that a complex network of neural interconnections between the PNS and SNS results in an elaborate system of ANS regulation and control of pancreatic insulin secretion (23).

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Studies of prolonged hyperglycemia due to chronic glucose infusion have found that enhanced insulin secretory responses to glucose are associated with changes in the ANS, through increases in parasympathetic activity and decreases in sympathetic activity (26). Therefore, adaptive changes in the ANS may contribute to altered insulin secretion by HC islets (34). The present studies evaluated the possibility that early changes in ANS stimulation of the HC rat pancreas, such as increased vagal tone or decreased sympathetic tone, contribute to the immediate onset of hyperinsulinemia in 12-day-old HC rats.

MATERIALS AND METHODS

Animal protocols. All animal protocols were approved by the Institutional Animal Care and Use Committee. Timed pregnant Sprague-Dawley rats were obtained from Zivic Miller Laboratories (Zellenpole, PA) and had access to a standard rodent laboratory chow (Harlan Tek-Lad, Madison, WI) and water ad libitum. The newborn pups were pooled and randomly assigned to each nursing mother (10 pups/dam) and then left with the mothers until postnatal day 4. On postnatal day 4, pups were assigned to control and experimental groups. In the mother-fed (MF) control group, pups were reared by their nursing mothers (caloric distribution in rat milk: 8% carbohydrate, 24% protein, and 68% fat), whereas pups in the high-carbohydrate (HC) experimental group were artificially reared on a HC formula (caloric distribution in HC milk formula: 56% carbohydrate, 24% protein, and 20% fat) (20). The high-fat (HF) control group was artificially reared on a HF milk formula with a caloric distribution similar to that of rat milk (caloric distribution in HF milk formula: 8% carbohydrate, 24% protein, and 68% fat). The artificial-rearing technique employed in this study has been described in detail previously (38). Briefly, intragastric cannulas (PE-10 polyethylene tubing) were placed while rat pups were under light anesthesia and individually housed in Styrofoam cups floating in 37°C water bath. Milk formula (HC or HF) was delivered for 20–25 min periods every 2 h at a rate of 0.45 kcal·g body wt⁻¹·day⁻¹ to match approximately MF calorie intake. The HC (or HF) milk formula was delivered via syringe pumps (PHD Syringe Pumps; Harvard Apparatus, Holliston, MA). The syringe pumps were connected to a multifunctional timer (Syrelec) which enabled them to be turned on for 20–25 min every 2 h. PE-50 polyethylene tubing was used for delivering the milk formula from the syringe pumps to the cannulas introduced in the rat pups. Pups were killed on postnatal day 12 and blood and pancreas were collected and processed as described below.

Postprandial plasma insulin levels in 12-day-old rats. Immediately after a morning feeding (9 AM), 12-day-old rat pups were given an intraperitoneal injection of saline, agonist or antagonist. After 10 min, pups were killed by decapitation, and trunk blood was collected in heparinized tubes. Plasma was separated by centrifugation and stored at −20°C. Plasma insulin levels were measured by radioimmunnoassay according to manufacturer’s instructions (Linco Research, St. Charles, MO). Dose-response experiments for acetylcholine chloride (ACh; cholinergic agonist; Sigma, St. Louis, MO) and oxymetazoline hydrochloride (OM; α2-adrenergic receptor-specific agonist; Sigma) were carried out as above with the following concentrations: ACh at 0.55, 2.75, and 13.75 μmol ACh/kg body wt and OM at 33.7, 168.5, and 674 nmol OM/kg body wt. Agonist and antagonist treatments for other experiments were as follows: ACh (2.75 μmol ACh/kg body wt), 4-diphenylacetoxy-N-methylpiperidino methobromide (4-DAMP; 0.21 μmol 4-DAMP/kg body wt; M3R antagonist; Sigma), yohimbine hydrochloride (Yoh; 10 μmol Yoh/kg body wt; α2-adrenergic antagonist; Sigma), or OM (33.7 nmol OM/kg body wt) (Fig. 1).

Isolation of islets from 12-day-old rats. Pancreatic islets were isolated from the 12-day-old rat pups by collagenase digestion as described previously (40). Briefly, pancreas from two rats were pooled and digested with collagenase (3 mg/preparation; type V; Sigma) and the islets were hand picked under a dissecting microscope.

Insulin secretion from islets isolated from 12-day-old rats. Islets were incubated in 500 μl of Krebs Ringer bicarbonate buffer (KRB). After preincubation, islets were incubated with 500 μl of fresh KRB buffer containing agonist/antagonist (as noted in figure legends) and desired glucose concentration (5.5 or 16.7 mM). Sample aliquots for determination of insulin secretion were taken at 10- and 60-min time points and stored at −20°C. Insulin levels were measured by radioimmunoassay according to manufacturer’s instructions. Dose-response experiments were carried out as described above using the following doses: ACh at 1, 10, and 100 μM and OM at 0.1, 1, and 10 μM. Agonist and antagonist treatments in specified experiments were as follows: 100 μM ACh, 100 μM 4-DAMP, 1 μM Yoh, or 1 μM OM.
Real-time polymerase chain reaction. RNA was isolated from islets of 12-day-old MF, HC, and HF rats using the TRIzol reagent-phenol-chloroform procedure (GIBCO-BRL, Rockville, CA) according to the manufacturer’s instructions. Levels of α2AR, M3R, phospholipase Cβ1 (PLCβ1), and protein kinase Cα (PKCα) mRNAs in pancreatic islets were measured via real-time RT-PCR using the iCycler system (Bio-Rad). Primer sequences, which were designed to span at least one exon-exon junction of the target mRNA to prevent amplification of contaminating genomic DNA, are described in Table 1. The mRNA levels detected by SYBR Green (Bio-Rad) analysis were normalized to 18S mRNA levels (QuantumRNA Classic II 18S Internal Standard, 324 bp; Ambion, Austin, TX). PCR efficiency was examined by serially diluting the template cDNA, and melting curve data were collected to assess PCR specificity. Each cDNA sample was run in triplicate, and a corresponding mRNA sample that had not been subjected to reverse transcription was included as a negative control in each run. Relative mRNA levels were calculated according to the comparative ΔΔCt method.

Statistical analysis. Results are expressed as means ± SE of six to eight independent experiments. Statistical analysis of experimental group vs. control group was done using Student’s t-test for MF and HC groups. One-way ANOVA was used to determine the significance of the difference of the means for MF, HC, and HF groups. This was followed by post hoc analyses using the Student-Newman-Keuls test. P values <0.05 were considered significant.

RESULTS

In vivo postprandial plasma insulin levels in 12-day-old rats. Plasma insulin levels were measured in 12-day-old rats during the immediate postprandial period after pretreatment with saline, agonist, or antagonist. Plasma insulin levels in 12-day-old HC rats treated with saline were significantly higher than age-matched MF and HF rats (Fig. 2A), whereas no significant differences in plasma insulin levels were seen between saline-treated MF and HF rats (Fig. 2A). These data indicate that the HC dietary intervention itself is responsible for changes in plasma insulin levels, and not the mode of feeding.

Dose-response experiments were carried out using three different concentrations of ACh (0.55, 2.75, and 13.75 μmol ACh/kg body wt) to determine the degree of responsiveness to cholinergic-induced potentiation of GSIS in 12-day-old HC and MF rats. Two types of comparisons (intergroup and intragroup) were made to analyze the results of these experiments. First, for each dose of ACh tested, the response of HC rats was higher in 12-day-old HC rats compared with age-matched MF and HF rats (Fig. 2A), whereas no significant differences in plasma insulin levels were seen between age-matched MF and HF rats treated with saline (Fig. 2B). Second, both MF and HC rats showed an increase in plasma insulin levels with increasing concentrations of ACh (Fig. 2B and Table 2). In addition, the increase in plasma insulin levels in HC rats in response to increasing concentrations of ACh relative to saline treatment (0 μM ACh) was significantly higher compared with similar analysis of MF rats (intragroup) (Table 2). While treatment of 12-day-old HF rats with 2.75 μmol ACh/kg body wt also resulted in increases in plasma insulin levels compared with saline-treated HF rats, no significant differences were seen between similarly treated 12-day-old MF and HF rats (Fig. 2A and Table 3).

Responses to blockade of in vivo cholinergic-induced potentiation of GSIS were evaluated using the M3R-specific antagonist 4-DAMP (0.21 μmol 4-DAMP/kg body wt). Treatment with 4-DAMP resulted in significantly lower plasma insulin levels in MF, HC, and HF rats compared with their respective saline treatments (Fig. 2A). In addition, the percent reduction in plasma insulin levels after treatment with 4-DAMP was significantly greater in 12-day-old HC rats compared with age-matched MF and HF rats (Table 3), suggesting that HC rats were more sensitive to cholinergic blockade of GSIS. No significant differences in plasma insulin levels were seen between age-matched MF and HF rats treated with 4-DAMP (Fig. 2A).

Dose-response experiments were carried out using three different concentrations of OM (33.7, 169, and 674 nmol OM/kg body wt) to determine the degree of sensitivity to adrenergic-induced inhibition of GSIS in 12-day-old HC and MF rats. Each concentration of OM significantly decreased plasma insulin levels in both MF and HC rats compared with saline-treated rats (Fig. 2C and Table 2). In addition, the percent decrease in plasma insulin levels compared with saline treatment were significantly less in 12-day-old HC rats compared with 12-day-old MF rats for each concentration of OM (Table 2). HF rats treated with 33.7 nmol OM/kg body wt also showed significantly reduced plasma insulin levels compared with saline-treated HF rats (Fig. 2A). No significant differences in plasma insulin levels were seen between 12-day-old MF and HF rats treated with 33.7 nmol OM/kg body wt (Fig. 2A).

Responses to blockade of in vivo adrenergic-induced inhibition of GSIS were evaluated using the α2-adrenergic receptor-specific antagonist Yoh (10 μmol Yoh/kg body wt). Treatment with Yoh resulted in significantly higher plasma insulin levels in MF, HC, and HF rats compared with their respective saline-treated rats (Fig. 2A). In addition, the percent increase of plasma insulin levels after Yoh treatment was significantly higher in 12-day-old HC rats compared with age-matched MF and HF rats (Table 3), suggesting that HC rats were more sensitive to adrenergic blockade of GSIS. No significant dif-

Table 1. Primers for real-time PCR

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2AR</td>
<td>198 bp (NM012739)</td>
<td>Sense, 5’-GCA TCA AGG CCA TCA TTG TCA-3’</td>
</tr>
<tr>
<td>M3R</td>
<td>172 bp (NM012527)</td>
<td>Antisense, 5’-CCA GGA TCA TGA GGC AAG G-3’</td>
</tr>
<tr>
<td>PLCβ1</td>
<td>213 bp (XM342524)</td>
<td>Sense, 5’-GAA CTT AGC CTT CGA CCT C-3’</td>
</tr>
<tr>
<td>PKCα</td>
<td>324 bp (X07286)</td>
<td>Antisense, 5’-GAC CCA AGC CAG ACC CAT-3’</td>
</tr>
</tbody>
</table>

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followed by post hoc analyses by Student-Newman-Keuls test for analyses of the significance of the differences between the 3 groups and this was increased by treatment with ACh in a dose-dependent manner with 5.5 mM glucose at 10 min (Fig. 3A) and 60 min (Fig. 3B) and with 16.7 mM glucose at 10 min (Fig. 3C) and 60 min (Fig. 3D) compared with their respective treatment with glucose alone (0 μM ACh). Treatment of HF islets with 100 μM ACh also resulted in significant increases of GSIS at 5.5 and 16.7 mM glucose compared with glucose treatment alone (Fig. 4, A-D). Furthermore, HC islets secreted significantly more insulin in response to each dose of ACh compared with similarly treated MF islets at both 5.5 mM (Fig. 3, A and B) and 16.7 mM (Fig. 3, C and D) glucose concentrations. In addition, the potentiation of GSIS in response to 100 μM ACh was significantly greater in HC islets at both 5.5 (Fig. 4, A and B) and 16.7 mM (Fig. 4, C and D) compared with MF and HF islets (Fig. 4; for percentage changes see Table 4). No significant differences in GSIS were seen between MF and HF islets treated with 100 μM ACh.

The effects of cholinergic blockade on GSIS by islets isolated from 12-day-old MF, HC, and HF rats at basal and stimulatory glucose concentrations were studied using 4-DAMP alone and in the presence of ACh. While treatment with 4-DAMP alone had no effect on GSIS by MF, HC, and HF islets at either glucose concentration, 4-DAMP in the presence of ACh significantly reduced ACh-induced potentiation of GSIS for all three groups at 5.5 and 16.7 mM glucose (Fig. 4; for percentage changes see Table 4). No differences were seen in insulin secretion between MF and HF islets treated with 4-DAMP or ACh in the presence of 4-DAMP (Fig. 4).

Previously, we showed that HC islets have reduced sensitivity to inhibition of insulin secretion by NE (34). Therefore, dose-response experiments were carried out using three different concentrations of OM (0.1, 1, and 10 μM) to determine the

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Change in Plasma Insulin Levels Compared with Saline Treatment</th>
<th>Ratio of Plasma Insulin Levels ( \text{HF/\text{MF}} \times 100 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>100 ± 2</td>
<td>293 ± 17</td>
</tr>
<tr>
<td>0.55 ACh</td>
<td>+40 ± 6*</td>
<td>350 ± 17</td>
</tr>
<tr>
<td>2.75 ACh</td>
<td>+92 ± 12*</td>
<td>426 ± 11</td>
</tr>
<tr>
<td>13.75 ACh</td>
<td>+171 ± 12*</td>
<td>456 ± 14</td>
</tr>
<tr>
<td>33.7 OM</td>
<td>−43 ± 4*</td>
<td>358 ± 6</td>
</tr>
<tr>
<td>169 OM</td>
<td>−57 ± 2*</td>
<td>380 ± 6</td>
</tr>
<tr>
<td>674 OM</td>
<td>−73 ± 3*</td>
<td>460 ± 10</td>
</tr>
</tbody>
</table>

Values are the results of 6–8 individual experiments and are represented as means ± SE. Plasma insulin levels in response to saline treatment were taken as 100% for each group. *P < 0.01 at least vs. saline treatment. MF, mother-fed; HC, high carbohydrate.

In vitro insulin secretion from islets isolated from 12-day-old rats. We evaluated the role of altered parasympathetic and sympathetic regulation of insulin secretion by HC islets in response to basal (5.5 mM) and stimulatory (16.7 mM) glucose. As previous studies have shown (1, 2), treatment with 5.5 and 16.7 mM glucose resulted in significantly greater insulin secretion by HC islets compared with control islets (Fig. 3).

Dose-response experiments were carried out using three different concentrations of ACh (1, 10, and 100 μM) to determine the sensitivity of islets isolated from 12-day-old HC and MF rats to cholinergic-induced potentiation of GSIS. Insulin secretion by MF and HC islets was significantly increased by treatment with ACh in a dose-dependent manner with 5.5 mM glucose at 10 min (Fig. 3A) and 60 min (Fig. 3B) and with 16.7 mM glucose at 10 min (Fig. 3C) and 60 min (Fig. 3D) compared with their respective treatment with glucose alone (0 μM ACh). Treatment of HF islets with 100 μM ACh also resulted in significant increases of GSIS at 5.5 and 16.7 mM glucose compared with glucose treatment alone (Fig. 4, A-D). Furthermore, HC islets secreted significantly more insulin in response to each dose of ACh compared with similarly treated MF islets at both 5.5 mM (Fig. 3, A and B) and 16.7 mM (Fig. 3, C and D) glucose concentrations. In addition, the potentiation of GSIS in response to 100 μM ACh was significantly greater in HC islets at both 5.5 (Fig. 4, A and B) and 16.7 mM (Fig. 4, C and D) compared with MF and HF islets (Fig. 4; for percentage changes see Table 4). No significant differences in GSIS were seen between MF and HF islets treated with 100 μM ACh.
sensitivity of islets isolated from 12-day-old HC and MF rats to adrenergic-induced inhibition of GSIS. Increasing concentrations of OM resulted in dose-dependent reductions in insulin secretion by MF and HC islets at both glucose concentrations compared with treatment with glucose alone (0 μM OM; Fig. 5). While each concentration of OM resulted in significant reductions of insulin secretion by MF islets, only the two highest doses of OM resulted in significant reductions in insulin secretion by HC islets compared with glucose treatment alone (0 μM OM; Fig. 5). Treatment with 10 μM OM also significantly reduced insulin secretion by HF islets at 5.5 and 16.7 mM glucose compared with glucose treatment alone (0 μM OM; Fig. 6). Furthermore, OM-induced reduction was significantly less in HC islets compared with MF and HF islets (Fig. 6; for percent changes see Table 4). MF and HF islets treated with 10 μM OM showed no significant differences in GSIS (Fig. 6).

The effects of adrenergic blockade on GSIS by islets isolated from 12-day-old MF, HC, and HF rats at basal and stimulatory glucose concentrations was studied using Yoh alone and in the presence of OM. While treatment with Yoh alone had no effect on GSIS by MF, HC, and HF islets at either glucose concentration (Fig. 6), Yoh in the presence of OM significantly attenuated adrenergic-induced inhibition at both glucose concentrations and resulted in significant normalization of insulin secretion by MF, HC, and HF islets with both 5.5 and 16.7 mM glucose at 10 and 60 min compared with treatment with OM alone (Fig. 6; for percent changes see Table 4).

**Islet mRNA levels in islets from 12-day-old rats.** The effects of the HC dietary intervention on cholinergic and adrenergic signaling pathways in pancreatic islets isolated from 12-day-old MF, HC, and HF rats were evaluated using quantitative real-time PCR. Levels of α2aAR mRNA were significantly reduced in HC islets (47%) compared with MF (100%) and HF islets (107; Fig. 7), which is consistent with previous data indicating that HC islets show reduced sensitivity to adrenergic-induced inhibition of insulin secretion. In addition, HC islets showed significant increases in M3R (260%), PLCβ1 (258%), and PKCα (233%) compared with MF (100% for M3R, PLCβ1, and PKCα) and HF islets (M3R = 109%; PLCβ1 = 114%; and PKCα = 115%; Fig. 7), which is consistent with previous data indicating that HC islets show increased sensitivity to cholinergic-induced potentiation of insulin secretion. No significant differences were seen between mRNA levels of MF and HF islets (Fig. 7).

**DISCUSSION**

The results from experiments presented in this report indicate that altered function of the ANS may play an influential role in the development of HC hyperinsulinemia in 12-day-old HC rats. In vivo and in vitro studies in 12-day-old HC rats found that significant increases in GSIS in response to cholinergic stimulation, as well as reduced sensitivity to adrenergic-induced inhibition of insulin secretion, were associated with hyperinsulinemia in these animals. Furthermore, increases in in islet expression of the cholinergic signaling molecules M3R, PLCβ1, and PKCα and decreases in the adrenergic receptor α2aAR provide further support that altered autonomic control of insulin secretion contributes to HC hyperinsulinemia.

**Table 3. Plasma insulin levels in MF, HC, and HF rats as a percentage of saline treatment**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MF</th>
<th>HC</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>100±2</td>
<td>100±2</td>
<td>100±2</td>
</tr>
<tr>
<td>2.75 ACh</td>
<td>+92±12*</td>
<td>+179±7*</td>
<td>+77±9*</td>
</tr>
<tr>
<td>0.21 4-DAMP</td>
<td>−37±5*</td>
<td>−62±3*</td>
<td>−34±5*</td>
</tr>
<tr>
<td>33.7 OM</td>
<td>−43±4*</td>
<td>−30±1*</td>
<td>−43±4*</td>
</tr>
<tr>
<td>1 Yoh</td>
<td>+103±10*</td>
<td>+183±14*</td>
<td>+102±8*</td>
</tr>
</tbody>
</table>

Values are the results of 6–8 individual experiments and are represented as means ± SE. Plasma insulin levels in response to saline treatment were taken as 100% for each group. *P < 0.01 at least vs. saline treatment. HF, high fat.
The dose-dependent increases in plasma insulin levels in response to ACh are consistent with other studies with adult animals, namely on HF diet (3) and with prolonged hyperglycemia (5, 28), showing that states of hyperinsulinemia and increased insulin demand are associated with increased parasympathetic activity and enhanced sensitivity to cholinergic stimulation of GSIS. Furthermore, the HC content of the HC dietary intervention results in an increase in the availability of glucose which has also been associated with increased parasympathetic activity and expression of M3R on rat β-cells (25, 30). The results obtained using the M3R-specific antagonist 4-DAMP showed that 12-day-old HC rats were more sensitive to 4-DAMP-mediated reduction in GSIS compared with controls. This finding is consistent with experiments of prolonged hyperglycemia in rats which have shown that hyperinsulinemia is dependent on increased parasympathetic activity and is reversed by treatment with the cholinergic antagonist atropine (5).

In a previous study in 12-day-old HC rats, we found that ACh treatment of HC islets resulted in greater insulin secretion in response to glucose compared with MF islets, suggesting increased β-cell responsiveness to cholinergic and incretin stimulation (34). The results from in vitro insulin secretory experiments using different concentrations of ACh showed potentiation of GSIS to be significantly higher in HC islets. These data are consistent with the observation that priming of

Table 4. Insulin secretion by MF, HC, and HF islets as a percent of saline treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group</th>
<th>5.5 mM Glucose</th>
<th>16.7 mM Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 min</td>
<td>60 min</td>
</tr>
<tr>
<td>None</td>
<td>MF</td>
<td>100±9</td>
<td>100±16</td>
</tr>
<tr>
<td></td>
<td>HC</td>
<td>100±7</td>
<td>100±16</td>
</tr>
<tr>
<td></td>
<td>HF</td>
<td>100±5</td>
<td>100±6</td>
</tr>
<tr>
<td>ACh (100 µM)</td>
<td>MF</td>
<td>+54±7*</td>
<td>+93±7*</td>
</tr>
<tr>
<td></td>
<td>HC</td>
<td>+112±8*</td>
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<tr>
<td></td>
<td>HF</td>
<td>+63±10*</td>
<td>+100±10*</td>
</tr>
<tr>
<td>ACh (100 µM) + 4DAMP (100 µM)</td>
<td>MF</td>
<td>+23±5</td>
<td>+28±5</td>
</tr>
<tr>
<td></td>
<td>HC</td>
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<td>HF</td>
<td>+30±6</td>
<td>+32±6</td>
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<td>OM (10 µM)</td>
<td>MF</td>
<td>−61±5*</td>
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</tr>
<tr>
<td></td>
<td>HC</td>
<td>−37±2*</td>
<td>−36±3*</td>
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<tr>
<td></td>
<td>HF</td>
<td>−59±4*</td>
<td>−60±5*</td>
</tr>
<tr>
<td>OM (10 µM) + Yoh (10 µM)</td>
<td>MF</td>
<td>−10±7</td>
<td>−16±5</td>
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<tr>
<td></td>
<td>HF</td>
<td>−10±6</td>
<td>−15±7</td>
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Values are the results of 6–8 individual experiments and are represented as means ± SE. Insulin secretion by saline-treated islets (no treatment) was taken as 100% for each time period and each glucose concentration. *P < 0.01 at least vs. saline treatment.
β-cells with ACh or the cholinergic agonist carbachol before a glucose challenge resulted in a higher potentiation of GSIS compared with unprimed β-cells (21, 42). Furthermore, treatment of HC islets with 4-DAMP in the presence of ACh resulted in significant reductions in cholinergic-induced insulin secretion, suggesting that ACh-induced potentiation of GSIS is mediated through activation of M3R as seen in a study showing that potentiation of GSIS by the cholinergic agonist oxotremorine M is inhibited by 4-DAMP (8).

Additionally, islets isolated from 12-day-old HC rats showed increased levels of M3R, PLCβ1, and PKCα mRNAs (~2.5-fold) compared with MF islets. Reductions in blood glucose levels, due to prolonged fasting of normal rats or prolonged ethanol consumption, have been associated with reduced expression of islet muscarinic receptors and reductions in cholinergic-induced insulin secretion (30). Conversely, streptozotocin-induced diabetic rats, which were chronically hyperglycemic, exhibited increases in expression of islet muscarinic receptors and cholinergic-induced potentiation of GSIS (30). Furthermore, reduced islet expression of PKCα and PKAα in response to protein deficiency during gestation and lactation is associated with impaired GSIS in offspring of...
protein-restricted rats (15, 16). Although plasma glucose levels are normal in 12-day-old HC rats, these studies suggest that the observed changes in cholinergic signaling pathways in HC rats may be due to altered gene expression of components of this pathway induced by the increased availability of glucose from the HC milk formula during the period of dietary intervention.

It is also of note that enhanced cholinergic signaling at the level of HC islets may be the result of a hyperactive cephalic phase of insulin secretion, which is consistent with previous findings that 12-day-old HC rats exhibited increased plasma GLP-1 levels and GLP-1 receptor levels in islets (34), as well as studies showing increased parasympathetic-induced GSIS associated with the incretin effect (4). Therefore, hyperinsulinemia in 12-day-old HC rats is associated with enhanced parasympathetic stimulation of GSIS, via programmed changes in cholinergic-induced insulin secretion in response to exposure to the early HC dietary intervention (Fig. 8).

$\alpha_{2a}$AR is the predominant $\alpha_2$-subtype expressed by $\beta$-cells and is responsible for adrenergic-induced inhibition of insulin secretion (29). In vivo experiments using the $\alpha_{2a}$AR-specific agonist OM found that 12-day-old HC rats exhibited reduced sensitivity to adrenergic-induced inhibition of insulin secretion compared with MF and HF rats. While progressive increases in OM concentrations resulted in greater reductions in plasma insulin levels in MF, HC, and HF rats, HC rats showed significantly lower reductions in GSIS compared with controls. Studies of offspring of rats fed a low-protein diet during gestation and lactation, which have shown that hypoinsulinemia is associated with increased sympathetic activity, are consistent with the idea that alterations in sympathetic activity can influence plasma insulin levels (26). Interestingly, treatment with Yoh, an $\alpha_{2A}$AR antagonist, resulted in a greater potentiation of plasma insulin levels in 12-day-old HC rats compared with controls, suggesting that HC rats are more sensitive to blockade of adrenergic-induced inhibition of GSIS. This appears to be inconsistent with previous experiments showing reduced sympathetic regulation of insulin secretion in HC rats. However, this may be due to treatment with a pharmacological dose of Yoh that acts on other systems in vivo, resulting in increased plasma insulin levels. For example, it is shown that the SNS is involved in regulation of parasympathetic activity, both centrally and peripherally (11, 12). Therefore, Yoh blockade of adrenergic inhibition of parasympathetic activity may result in increased cholinergic-induced potentiation of insulin secretion.

**Fig. 7.** Quantitative real-time PCR analysis of $\alpha_{2a}$AR, M3R, PLC$\beta_1$, and PKCa mRNAs in pancreatic islets isolated from 12-day-old MF (open bars), HC (filled bars), and HF rats (gray bars). Threshold cycle values were normalized using 18S as an internal standard and fold-difference was calculated using the comparative $C_T$ method ($\Delta\Delta C_T$). Individual samples were run in triplicate and values represent means ± SE of 7 independent experiments. ANOVA was performed to compare the significance of the difference of the mRNA levels of MF, HC, and HF islets. Post hoc analyses were performed using the Student-Newman-Keul’s test. *$P < 0.01$ vs. MF mRNA levels.

**Fig. 8.** Summary of study findings related to $\beta$-cell insulin secretion pathways. Increased parasympathetic activity, through increased sensitivity to cholinergic stimulation and increased levels of M3R, PLC-$\beta_1$, and PKCa mRNAs, results in enhanced insulin secretion in response to glucose by $\beta$-cells from 12-day-old HC rats. In addition, reduced sensitivity to sympathetic stimulation results in further increases in insulin secretion, in association with decreased $\alpha_{2a}$AR mRNA levels.
Previous in vitro studies from our laboratory also showed that islets from 12-day-old HC rats exhibited reduced sensitivity to NE-mediated inhibition of insulin secretion, as seen with the 10-fold higher concentration of NE required to completely inhibit GSIS compared with MF islets (34). Reduced sensitivity of HC rats to adrenergic stimulation is consistent with other studies showing that stimulation of SNS activity induces inhibition of HC islets from 12-day-old HC rats exhibiting reduced sensitivity to adrenergic-induced inhibition of GSIS and is consistent with other studies showing regulation of adrenergic activity by metabolic signals. For example, increased cAMP signaling, due to increased adenylate cyclase activity, stimulation with the adenylate cyclase activator forskolin or the membrane-permeable cAMP analog dibutyryl cAMP, has been shown to reduce $\alpha_2\beta$AR expression (32). Reduction of $\alpha_2\beta$AR expression in HC islets by increased cAMP levels is consistent with previous studies showing increased mRNA levels of adenylate cyclase type VI and PKA activity (34), which are indicative of increased levels of cAMP. Therefore, altered expression of cholinergic and adrenergic signaling molecules indicates that altered autonomic activity in HC rats may play an important role in programming of hyperinsulinemia in response to the HC dietary intervention (Fig. 8).

In conclusion, altered autonomic activity contributes to enhanced $\beta$-cell insulin secretory responses to glucose stimulation through increased parasympathetic and decreased sympathetic activity in HC rats. The fact that changes in autonomic activity are present during the suckling period suggests that the overlap of the HC dietary intervention with continued postnatal development of the nervous system results in altered autonomic regulation of insulin secretion. Further studies pertaining to the metabolic programming in response to early nutritional interventions will advance our understanding of the growing obesity and type 2 diabetes epidemics, which have been associated, in part, with metabolic programming phenomenon.

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