Hexosamines regulate sensitivity of glucose-stimulated insulin secretion in β-cells

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Cooksey, Robert C., Sumitha Pusuluri, Mark Hazel, and Donald A. McClain. Hexosamines regulate sensitivity of glucose-stimulated insulin secretion in β-cells. Am J Physiol Endocrinol Metab 290: E334–E340, 2006. First published September 27, 2005; doi:10.1152/ajpendo.00265.2005.—Hexosamines serve a nutrient-sensing function through enzymatic O-glycosylation of proteins. We previously characterized transgenic (Tg) mice with overexpression of the rate-limiting enzyme in hexosamine production, glutamine:fructose-6-phosphate amidotransferase, in β-cells. Animals were hyperinsulinemic, resulting in peripheral insulin resistance. Glucose tolerance deteriorated with age, and males developed diabetes. We therefore examined islet function in these mice by perfusion in vitro. Young (2-mo-old) Tg animals had enhanced sensitivity to glucose of insulin secretion. Insulin secretion was maximal at 20 mM and half maximal at 9.9 ± 0.5 mM glucose in Tg islets compared with maximal at 30 mM and half maximal at 13.5 ± 0.7 mM glucose in wild type (WT; P < 0.005). Young Tg animals secreted more insulin in response to 20 mM glucose (Tg, 1.254 ± 0.11; WT, 0.45 ± 0.23 pg/islet·1·35 min−1; P < 0.01). Islets from older (8-mo-old) Tg mice became desensitized to glucose, with half-maximal secretion at 16.1 ± 0.8 mM glucose, compared with 11.8 ± 0.7 mM in WT (P < 0.05). Older Tg mice secreted less insulin in response to 20 mM glucose (Tg, 2.256 ± 0.42; WT, 3.493 ± 0.367 pg/islet·1·35 min−1; P < 0.05). Secretion in response to carbobach was similar in WT and Tg at both ages. Glucose oxidation was blunted in older Tg islets. At 5 mM glucose, islet CO2 production was comparable between Tg and WT. However, WT mice increased CO2 production 2.7 ± 0.4-fold in 20 mM glucose, compared with only 1.4 ± 0.1-fold in Tg (P < 0.02). Results demonstrate that hexosamines are involved in nutrient sensing for insulin secretion, acting at least in part by modulating glucose oxidation pathways. Prolonged excess hexosamine flux results in glucose desensitization and mimics glucose toxicity.

islet; desensitization; glucose sensing

GLUCOSE METABOLISM through the hexosamine biosynthesis pathway serves as a nutrient-sensing function and has been shown to mediate many of the adverse effects of hyperglycemia, or so-called “glucose toxicity.” This hypothesis has been supported by studies in diverse experimental systems, including cultured cells and intact animals treated with either glucosamine or genetically engineered alterations in the hexosamine signaling pathway (3–5, 7, 10–12, 21, 29, 32, 37). The rate-limiting enzyme in the hexosamine biosynthesis pathway is glutamine:fructose-6-phosphate amidotransferase (GFA), which catalyzes the formation of glucosamine 6-phosphate from fructose 6-phosphate and glutamine. The terminal metabolites of the hexosamine pathway are uridyl diphosphate (UDP)-hexosamines. UDP-N-acetylglucosamine (UDP-GlcNAc) is a substrate for the cytosolic UDP-GlcNAc peptide glycosyltransferase [or O-glycosyltransferase (OGT)], which glycosylates nuclear and cytosolic proteins with a single N-acetylglucosamine moiety on serine and threonine residues (14, 20). Recently, this O-linked glycosylation (O-GlcNAc) has been shown to mediate the effects of stimulating the hexosamine pathway (23, 28, 38).

A pattern of results in these experimental model systems is that hexosamines signal a shift in gene expression and enzymatic activities that favor long-term energy storage in the organism. Over time, many of the model animals also develop characteristics of the type 2 diabetes syndrome. We have previously reported (35) that overexpression of GFA targeted to β-cells results in peripheral hyperinsulinemia. In response, the animals become modestly insulin resistant, and with age the male mice develop diabetes. To further explain these findings, we have performed studies on isolated islets from the mice expressing GFA under control of the rat insulin promoter (RIP). We demonstrate that hexosamine flux initially sensitizes the cells to glucose-induced insulin secretion, but as the animals age, the cells become desensitized. This is associated with a defect in glucose oxidation in the islets.

RESEARCH DESIGN AND METHODS

Materials. Routine reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Transgenic animals. Transgenic mice on a C57BL6 background with GFA targeted to pancreatic β-cells with the RIP have been described previously (35). Mice that were heterozygous for the transgene were compared with nontransgenic wild-type mice as controls. Mice were genotyped by polymerase chain reaction using transgene-specific primers. All procedures were approved by the Institutional Animal Care and Use Committee.

Isolation of islets. Islets of Langerhans were isolated from fasted wild-type and transgenic mice by collagenase digestion (Roche Applied Science, Indianapolis, IN), as described in the kit instructions. The islets were then individually handpicked from acinar tissue.

Islet perfusion. Islets were perfused as previously described (45, 46). Briefly, groups of size-matched islets (10–15/group) were isolated and placed on a 62-µm monofilament nylon mesh (Small Parts, Miami Lakes, FL) and inserted in 13-mm filter holders (Swinnex, Millipore, Bedford, MA). Islet groups were perfused (1 ml/min) in Krebs-Ringer bicarbonate buffer (NaCl, 126 mM; KCl, 4.8 mM; KH2PO4, 1.2 mM; MgSO4·7H2O, 1.2 mM; CaCl2, 2.2 mM; NaHCO3, 5.0 mM; HEPES, 10 mM; glucose, 2.8 mM; BSA, 1%, pH 7.4). The buffer was gassed with 95% O2-5% CO2 and maintained at 37°C. After a 30-min equilibration period, the islet groups were perfused with 3, 5, 10, 20, or 30 mM glucose or 10 mM glucose plus 10 µM carbobach and 1-m fractions of the perfusate collected for an additional 40 min. Insulin concentration in the perfusate was determined by radioimmunoassay (Sensitive Rat Insulin RIA Kit; Linco Research, St. Charles, MO).

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Quantitative RT-PCR analysis of mRNA. Between 20 and 72 isolated islets per mouse from six transgenic and five wild-type (sibling), C57BL/6-strain background, 8-mo-old male mice were collected, submerged in 250 μl of RNA-Later (Ambion, Austin, TX), and stored at −20°C. Each islet sample was diluted with 200 μl of neutral PBS and centrifuged (2,500 rcf for 1 min at 4°C). The supernatant was gently removed, and 250 μl of TRIzol Reagent (MRC, Cincinnati, OH) were added. RNA isolation steps were performed according to the manufacturer’s protocol, except that the tissue was homogenized using a Sonic Dismembrator 60 (Fischer Scientific, setting 2.5 for 24 × 0.5-s pulses). Each RNA pellet was suspended in 40 μl of FORMazol (stabilized formamide, MRC). RNA yields were between 1.1 and 2.8 μg/sample. First-strand cDNA synthesis used Supplementary II reverse transcriptase (Invitrogen Life Technologies), according to that manufacturer’s protocol, with 380 ng of islet RNA as described (16).

Quantitative PCR was performed with a Roche LightCycler using Primer3-designed primers as previously described (4), except that the cycle’s annealing step was set at 60°C for all primers. Six nanograms of cDNA (based on an assumed 1:5 mass ratio of RNA input to cDNA output during cDNA synthesis with Superscript II reverse transcriptase (Invitrogen Life Technologies), according to that manufacturer’s protocol, with 380 ng of islet RNA as described (16). The transgenic islets. In wild-type islets, maximal insulin secretion was increased by 10.5 ± 2.3 μU/ml (mean ± SE, n = 3 mice; 10 mice studied per group).

Table 1. Islet mRNA levels in WT and RIP-GFA Tg mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>mRNA (Tg/WT)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDHA</td>
<td>1.38</td>
<td>0.11</td>
</tr>
<tr>
<td>SDHB</td>
<td>0.91</td>
<td>0.43</td>
</tr>
<tr>
<td>Aco2</td>
<td>0.89</td>
<td>0.58</td>
</tr>
<tr>
<td>Cis</td>
<td>0.99</td>
<td>0.95</td>
</tr>
<tr>
<td>MDH</td>
<td>1.02</td>
<td>0.87</td>
</tr>
<tr>
<td>Ndua</td>
<td>1.00</td>
<td>0.97</td>
</tr>
<tr>
<td>Ndu1b</td>
<td>1.03</td>
<td>0.66</td>
</tr>
<tr>
<td>ATP5e</td>
<td>1.06</td>
<td>0.43</td>
</tr>
<tr>
<td>GLUT2</td>
<td>0.91</td>
<td>0.49</td>
</tr>
<tr>
<td>ATP6</td>
<td>1.10</td>
<td>0.74</td>
</tr>
<tr>
<td>Ins1</td>
<td>1.96</td>
<td>0.79</td>
</tr>
<tr>
<td>Ins2</td>
<td>0.84</td>
<td>0.18</td>
</tr>
</tbody>
</table>

RIP-GFA, rat insulin promoter glutamine-fructose-6-phosphate amidotransferase; Tg, transgenic; WT, wild type; SDHA, succinate dehydrogenase A; SDHB, succinate dehydrogenase B; Aco2, aconitate; Cis, citrate synthase; MDH, malate dehydrogenase; Ndua, NADH-ubiquinone oxidoreductase MWFE subunit; Ndu1b, NADH-ubiquinone oxidoreductase B22 subunit; ATP5e, ATP synthase subunit-e; GLUT2, glucose transporter 2; mitochondria-encoded ATP synthase subunit; Ins1, insulin 1; Ins2, insulin 2; n = 5–6 for each group.

The results summarized above suggest altered regulation of insulin secretion from β-cells exposed to excess hexosamine flux. To examine this further, we isolated islets from normal and transgenic mice and studied insulin secretion at various glucose concentrations. Islets from young (2-mo-old) mice exhibited hyperinsulinemia (64–65% higher serum insulin levels in the fasting and post-challenge states) with normal fasting glucose and slightly lower glucose excursions after intraperitoneal glucose challenge than their wild-type littermates. Older (8–10 mo) transgenic females exhibited a slight trend toward higher glucose excursions, whereas in the transgenic males the glucose levels during an intraperitoneal glucose tolerance test were significantly higher than in their wild-type littermates. Weight loss was observed with the transgenic mice when aged. In the face of the deterioration of glucose tolerance, insulin levels did not increase in the older animals.

Insulin secretion in isolated islets. The results summarized above suggest altered regulation of insulin secretion from β-cells exposed to excess hexosamine flux. To examine this further, we isolated islets from normal and transgenic mice and studied insulin secretion at various glucose concentrations. Islets from young (2-mo-old) mice exhibited enhanced insulin secretion at 20 mM glucose (Fig. 1A). Significantly more insulin was secreted in both the first and second phases, with the incremental area under the curve threefold higher in islets from the RIP-GFA transgenic mice (Fig. 1B). Both the first and second phases of insulin secretion were similarly increased. Insulin secretion was examined at various concentrations of glucose and is presented as the fold increase over basal secretion as a function of glucose concentration (Fig. 1C). The sensitivity for glucose-induced insulin secretion was greater in the transgenic islets. In wild-type islets, maximal insulin se-
cretion was reached at 30 mM glucose, and half-maximal secretion at 13.5 ± 0.7 mM glucose. In RIP-GFA islets with increased hexosamine flux, secretion was maximal at 20 mM and half-maximal at 9.9 ± 0.5 mM glucose (Fig. 1D, *P < 0.05). These results differ from those originally published by our group (35) in that the islet insulin content was originally reported to be higher in the older transgenic mice. We do not know fully the reason for this discrepancy, but we have noted a loss over time of copy number of the concatamerized transgene (not shown).

In contrast with islets from 2-mo-old mice, islets from older (8-mo-old) mice exhibited decreased insulin secretion and glucose desensitization. Insulin secretion was 35% lower at 20 mM glucose in islets from the RIP-GFA transgenic islets (*P < 0.05; Fig. 3, A and B). Sensitivity to glucose of insulin secretion was also lower in the older transgenic islets. Half-maximal secretion occurs at 16.1 mM glucose in RIP-GFA islets compared with 11.8 mM in wild types (*P < 0.05; Fig. 3, C and D). At very high concentrations of glucose, insulin secretion decreased in both wild-type and transgenic islets, but the transgenic islets, in addition to being less sensitive to glucose-induced insulin secretion, were also desensitized to this adverse effect of high glucose. In wild-type mice, secretion fell to 64% of normal at 40 mM glucose but only 82% of normal in transgenic islets (*P < 0.05). Insulin content of the islets in the older mice tended to be lower than in wild types, but not significantly so (Fig. 2). Of note, all of the perifusion studies employed islets from both male and female mice, and no differences were noted in insulin secretion as a function of sex. Thus the abnormalities seen in the islets are likely not a function of a secondary response to hyperglycemia, which develops significantly only in male mice (35).

Fig. 2. Insulin content of islets from wild-type and RIP-GFA mice. Islets were collected as for perifusion study. Groups of 4–5 islets were sonicated, and insulin concentration measured (n = 8–10 independent determinations/group).
Pharmacological stimulation of insulin secretion. To determine whether there was a general defect in insulin secretion, islets were exposed to carbachol. There were no significant differences between transgenic and wild-type islets in levels of carbachol-induced insulin secretion (Fig. 4).

Glucose oxidation in isolated islets. The reduced and desensitized insulin secretion observed in the islets of older transgenic mice in response to glucose, but not carbachol, suggests a defect in a more proximal step in glucose sensing. Islets from older mice exhibited a significant decrease in glucose oxidation (Fig. 5). At 5 mM glucose, CO₂ production in 8-mo-old islets was comparable between transgenic and wild-type mice (wild type 0.76 ± 0.14, transgenic 0.89 ± 0.08 pmol CO₂/100islets/h). However, islets from wild-type mice increased CO₂ production 2.7 ± 0.4-fold in 20 mM glucose compared with 5 mM glucose, whereas in transgenic islets the increase was only 1.4 ± 0.1-fold (P < 0.02).

Assays of select mitochondrial enzyme activities and mRNAs. We next investigated whether the observed decrease in glucose oxidation might be related to changes in mRNAs for proteins either known to be involved in glucose oxidation and deregulated in diabetes or for proteins previously demonstrated to be regulated by hexosamine flux. Several mRNAs for nuclear and mitochondrially encoded enzymes were unaltered in the transgenic islets (Table 1). The list includes several proteins previously shown to be downregulated in the fat cells of glucosamine-treated rats (MDH, Ndu1a, Ndu1b, ATP5e) (26). Enzyme activities of citrate synthase, succinate dehydrogenase, and pyruvate carboxylase were also unaltered (data not shown).

DISCUSSION

The hallmarks of β-cell dysfunction in type 2 diabetes include early hypersecretion, thought to be a response to insulin resistance, followed by progressive loss of glucose-

Fig. 3. Perifusion of islets from 8-mo-old mice. Studies shown in Fig. 1 for 2-mo-old mice were repeated using islets from 8-mo-old mice. Islets from male and female mice were used, with no differences detected as a function of sex. A: 4–5 islets from control or RIP-GFA transgenic mice were exposed to 3 mM glucose in perifusion chambers. At 35 min, glucose concentration was increased to 20 mM. Fractions were collected every minute for 10 min and then every 5 min thereafter; n = 6/group. B: quantification of data from A. For each perifusion, incremental area under insulin curve above baseline (average value for 3 mM glucose used as baseline) was calculated (n = 6/group; *P < 0.05). C: glucose dose response of insulin secretion. Perifusions were repeated at 10, 30, and 40 mM glucose (n = 4–6 each). Results are plotted as fold increase over basal (3 mM glucose) secretion for each glucose concentration. D: glucose concentration leading to ED50 of insulin secretion. Data in C were plotted as %maximal secretion, and a regression line was calculated. Interpolated value of glucose at 50% maximal secretion was then determined (*P < 0.05).

Fig. 4. Carbachol-stimulated insulin secretion in control and RIP-GFA islets. Perifusion studies were carried out as described in legends to Figs. 1 and 2, but at 35 min, 10 mM glucose and 10 μM carbachol were added to perifusion buffer. A: 2-mo-old mice (n = 4/group). B: 8-mo-old mice (n = 4/group).
induced insulin secretion and ultimate loss of β-cell mass. Many of these features are also seen in β-cells exposed to high concentrations of glucose (31). The molecular mechanisms for this so-called “glucose toxicity” are unknown, but we have shown previously (35) that some of these effects can be reproduced in islets that overexpress GFA, which is the rate-limiting enzyme in hexosamine synthesis. In the present work, we have extended these observations and reported that excess hexosamine flux results initially in augmentation of glucose-induced insulin secretion, but with aging there is progressive desensitization of insulin secretion accompanied by a defect in glucose oxidation. The hexosamine pathway of glucose metabolism also contributes to insulin resistance (3, 5–7, 10, 12, 21, 29, 32), obesity (11, 37), and hyperlipidemia (37) and has been hypothesized to function as a cellular satiety sensor responsible for damping acute insulin signaling and facilitating the storage of excess nutrients as fat (22, 30, 41). The pathway exerts its effects through the substrate-limited O-GlcNAc of nuclear and cytosolic proteins by the enzyme OGT (23, 28, 38).

There have been several studies that have addressed the effects of hexosamine flux on β-cells. Shankar et al., for example, showed that glucosamine infusion into intact rats led to a blunting of both glucose- and arginine-stimulated insulin secretion (34). It was later shown that glucosamine treatment or GFA overexpression in isolated rat islets also resulted in decreased insulin secretion and downregulation of insulin gene expression (13). In the latter case, however, an inhibitor of enzymatic deglycosylation did not mimic GFA overexpression, leading the authors to conclude that O-GlcNAc did not mediate the effects. Rather, it was found that glucosamine could increase hydrogen peroxide levels, leading the authors to conclude that oxidant stress was responsible. Another report (50) demonstrated that inhibition of the hexosamine pathway led to decreased insulin secretion from mouse islets, suggesting that the pathway might trigger biphasic responses (stimulatory and inhibitory) on insulin secretion depending on the degree or chronicity of stimulation. Other reports (1, 8, 18) have demonstrated β-cell death and mitochondrial dysfunction in glucosamine-treated β-cells. Some caution should be used in interpreting the studies using glucosamine exposure, however, in that glucosamine in high concentrations can inhibit glucokinase and glucose transporter 2 (GLUT2)-mediated glucose transport (2, 36). Overexpression of GFA, by contrast, would not be expected to generate significant amounts of free glucosamine and has not done so in animal models (12). One possible complication of the present study is the known expression of insulin promoter-targeted genes in the hypothalamus, although this should have relatively little impact on the phenotype of the isolated islets.

The mechanism for the decrement of glucose-induced insulin secretion in older islets that overexpress GFA likely involves decreased glucose oxidation. Beyond that result, however, the detailed molecular mechanism remains unclear. It has been reported that hexosamine flux can increase the DNA-binding activity of the transcription factor pancreatic duodenal homeobox-1 (PDX-1) (9) that includes as its targets the genes for glucokinase, GLUT2, and insulin (17, 24, 27, 39, 40). This represents one mechanism by which the increase in glucose-stimulated insulin secretion might occur in the younger animals.
The older transgenic animals exhibit a loss of glucose-induced insulin secretion, and an obvious question arises as to whether both contradictory responses could be mediated by the same hexosamine pathway. The same genes whose expression would be stimulated by glycosylation of PDX-1 have also been observed to be downregulated in islets with high hexosamine flux (13, 44). Consistent with this, we also observed a trend toward downregulation of GLUT2 and the insulin2 gene in the older transgenic islets. The modest nature of these changes might be related to the relatively low degree of GFA overexpression in the islets (35). In animal models of GFA overexpression in several tissues, we have experienced a consistent difficulty in achieving high levels of overexpression, which is perhaps related to the proapoptotic effects of the pathway (8, 18, 19, 25, 42). This might also explain the lack of significant decreases in the levels of message for several other mRNAs reported to be downregulated by the hexosamine pathway (26), although those effects might also be restricted to the adipocytes where the phenomenon was observed. Other defects, such as impaired phosphoinositide signaling, have also been observed in β-cells treated with glucosamine (47). Thus it is likely that a panoply of effects mediated by transcriptional regulation and posttranslational modification of proteins by O-GlcNAc may contribute to the observed decrease in β-cell function induced by high hexosamine flux. This would not be surprising, given the hundreds of proteins that are known to be modified by O-GlcNAc (43). The breadth and complexity of the response to O-GlcNAc might also explain the diverse results that were observed as a function of whether the increased hexosamine flux is acute or chronic or of large or small magnitude. As one example of the competing and disparate effects of hexosamine signaling, overexpression of the pathway has been reported to both induce oxidant stress (13) and protect from oxidant stress (49). Such apparently contradictory effects could be rationalized teleologically; if the hexosamine pathway is a sensor for high nutrient fluxes, then a rational response to those fluxes would be to upregulate the capacity for mitochondrial oxidation while at the same time upregulating antioxidant defense mechanisms. The net result in any tissue would then reflect the oxidant burden vs. the degree of upregulation of antioxidant defenses. These would presumably vary across tissues, with nutrient burden and, perhaps as in the present study, with the age of the host. At this time, however, we cannot rule out an alternative hypothesis, namely that the primary effect of the hexosamine pathway is glucose sensitization, whereas the desensitization is a secondary response, for example, to the hyperglycemia or insulin resistance that develops in the model. The latter hypothesis is made less likely by the finding that the islets from both male and female mice exhibit the glucose-insensitive phenotype, even though the female mice exhibit only very mild glucose intolerance (35).

In summary, we have demonstrated that hexosamine flux in β-cells exerts a biphasic effect, initially stimulating but then blunting glucose-induced insulin secretion through the inhibition of glucose oxidation. These effects parallel features of type 2 diabetes and suggest that excess nutrient flux may exert some of their adverse effects through this pathway. This hypothesis is supported by the observed association with diabetes of polymorphisms in genes encoding key enzymes in the hexosamine pathway (15, 48).

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