In vivo and in vitro evidence that chronic activation of the hexosamine biosynthetic pathway interferes with leptin-dependent STAT3 phosphorylation

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Zimmerman AD, Harris RB. In vivo and in vitro evidence that chronic activation of the hexosamine biosynthetic pathway interferes with leptin-dependent STAT3 phosphorylation. Am J Physiol Regul Integr Comp Physiol 308: R543–R555, 2015. First published January 7, 2015; doi:10.1152/ajpregu.00347.2014.—We previously reported that a 2-day peripheral infusion of glucosamine caused leptin resistance in rats, suggesting a role for the hexosamine biosynthetic pathway (HBP) in the development of leptin resistance. Here we tested leptin responsiveness in mice in which HBP activity was stimulated by offering 30% sucrose solution in addition to chow and water or by infusing glucosamine. Mice were leptin resistant after 33 days of access to sucrose. Resistance was associated with increased activity of the HBP and with phosphorylation of transcription factor signal transducer and activator of transcription-3 Tyr705 [pSTAT3(Y705)] but inhibition of suppressor of cytokine signaling 3 in the liver and hypothalamus. Intravenous infusion of glucosamine for 3 h stimulated pSTAT3(Y705) but prevented leptin-induced phosphorylation of STAT3(S727). In an in vitro system, glucose, glucosamine, and leptin each dose dependently increased O-linked β-N-acetylgalactosamine (O-GlcNAc) protein and pSTAT3(Y705) in HepG2 cells. To test the effect of glucose on leptin responsiveness cells were incubated in 5.5 mM (LG) or 20 mM (HG) glucose for 18 h and were treated with 0 or 50 ng/ml leptin for 15 min. HG alone and LG + leptin produced similar increases in O-GlcNAc protein, glutamine fructose-6-phosphate amidotransferase (GFAT), and pSTAT3(Y705) compared with LG media. Leptin did not stimulate these proteins in HG cells, suggesting leptin resistance. Leptin-induced pSTAT3(S727) was prevented by HG media. Inhibition of GFAT with azaserine prevented LG + leptin and HG stimulation of pSTAT3. These data demonstrate development of leptin resistance in sucrose-drinking mice and provide new evidence of leptin-induced stimulation of the HBP.

sucrose solution; HepG2 cells; pSTAT3; O-GlcNAc

The cytokine leptin is released primarily from white adipose tissue and is hypothesized to function as a negative feedback signal in the control of energy balance (52) by inhibiting food intake and causing weight loss in normal weight animals (12, 14). We have previously shown that rats fed a choice diet of chow, lard, and 30% sucrose solution are leptin resistant within 3 wk of being offered the diet (13) and that this resistance is rapidly reversed once they are switched back to chow (1). Rats fed the choice diet or chow plus 30% liquid sucrose develop leptin resistance more quickly than rats fed a high-fat diet, rats fed chow plus lard, or rats consuming the same amount of sucrose from a dry diet, even though the high-fat-fed and chow plus lard rats are as fat as the rats offered chow plus sucrose (13). These results suggest that the consumption of sucrose solution causes changes in metabolism that disrupt leptin signaling.

Activity of the hexosamine biosynthetic pathway (HBP) increases in response to increased substrate availability. Therefore, the HBP has been proposed to serve as a nutrient sensor that can modify metabolism and energy expenditure (35). Once glucose enters a cell it is rapidly converted to glucose-6-phosphate and then fructose-6-phosphate, a majority of which enters glycolysis. Normally, 1–3% of the fructose-6-phosphate enters the HBP (27). When nutrient availability is increased, a greater percentage of the fructose-6-phosphate is shunted to the HBP where it is converted into glucosamine-6-phosphate by glutamine fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme for glucose entry into the HBP (5, 29).

The product of the pathway, uridine 5′-diphospho-N-acetylgalactosamine (UDP-GlcNAc), is the substrate for O-linked β-N-acetylgalactosamine (O-GlcNAc) modification of serine or threonine residues of cytosolic and nuclear proteins. This reversible reaction is catalyzed by O-GlcNAc transferase (OGT) (45). The removal of O-GlcNAc is dependent on the enzyme β-d-N-acetylgalactosaminidase (O-GlcNAcase) (25, 51). Hundreds of proteins critical to the cell cycle, embryonic survival, the cellular stress response, and many other cellular functions are O-GlcNAc modified (25, 47, 51).

Previously, we reported that a 2-day peripheral infusion of glucosamine (GlcN), which enters unregulated into the HBP, causes leptin resistance in rats (41), suggesting a direct effect of HBP activation on leptin responsiveness. The objective of the current studies was to further clarify the relation among consumption of sucrose solution, activation of the HBP, and leptin resistance and to develop an in vitro model to study the direct effect of HBP activation on cellular leptin responsiveness. We used human hepatoma HepG2 cells for the in vitro studies because they have been shown to express a functional HBP (17) and the long-form leptin receptor (ObRb) (54). We did not test the response to fructose because liver is the only tissue that metabolizes any appreciable amount of fructose and glucose is the substrate driving the HBP for a majority of leptin responsive tissues. ObRb activation initiates phosphorylation of transcription factor signal transducer and activator of transcription-3 (STAT3) (3), phosphoinositide 3-kinase (PI3K) (33), and extracellular-signal-regulated kinases 1/2 (ERK1/2) (36). Leptin stimulation of ObRb also causes a rapid increase in the expression of suppressor of cytokine signaling-3 (SOCS3), which inhibits further STAT3 activation (32). Disruption of the STAT3 binding site on ObRb in mice leads to a reduced energy expenditure, overeating, and a degree of obesity equivalent to that in mice that lack ObRb (3); therefore, phosphorylation of STAT3(Y705) is a commonly used marker for leptin responsiveness (40) and was used as such in the experiments described here.
METHODS

The C57BL/6J mice used in these studies were obtained from a colony maintained at Georgia Regents University. Unless specified otherwise, the mice were group housed in shoe-box cages in rooms maintained at 70°F, 50% humidity with lights on 12 h/day from 7:00 AM. All animal procedures were conducted in accordance with accepted standards of humane care in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Georgia Regents University.

Experiment 1: effect of consumption of sucrose solution on leptin and insulin response in mice. Ten 8-wk-old male mice were offered free access to chow and water or chow, water, and 30% sucrose solution. After 30 days the mice were moved into individual cages with grid floors to allow measurement of food and sucrose consumption. On day 33 the mice were food deprived from 7:00 AM to 5:00 PM. Half of the animals received an intraperitoneal injection of PBS and half received 1 mg leptin/kg (mouse recombinant leptin; R&D Systems, Minneapolis, MN). Food and sucrose were returned to the cages at 6.00 PM, and energy intake and body weight were measured 14, 38, and 62 h after injection. The mice received a second injection 24 h after the first injection but were not food deprived before this second injection. The leptin test was repeated on days 38 and 39 with treatments reversed so that mice injected with PBS on days 33 and 34 were injected with leptin on days 38 and 39 and vice versa. On day 42 an insulin tolerance test (ITT) was performed. The mice were food deprived for 5 h from 7:00 AM, and then blood glucose was measured on a small tail blood sample using glucose strips (EasyGluco Plus; US Diagnostics, New York, NY). Each mouse was injected intraperitoneally with 2 mU insulin/kg (Humulin Insulin; Eli Lilly, Indianapolis, IN), and blood glucose was measured 10, 20, 30, 40, and 50 min after injection. The data are means ± SE for groups of 5 mice. *Significant differences between chow and sucrose mice or between PBS and leptin injected chow mice during the leptin test.
injection before food was returned to the cage. On day 44 mice were decapitated in the morning after 2 h of food deprivation, and blood was collected for measurement of serum leptin, insulin, and glucose. White fat depots and liver were dissected and weighed. One lobe of the liver was snap frozen and used for Western blot detection of pSTAT3(Y705), pSTAT3(S727), STAT3, SOCS3, O-GlcNAc, GFAT, OGT, O-GlcNAcase (see below).

A second group of male mice were offered either chow and water or chow, water, and 30% sucrose solution. On day 34 they were decapitated in afternoon after 5 h of food deprivation. Blood was collected for measurement of glucose, insulin, and leptin. Liver was dissected, weighed, and snap frozen. The brain was collected, and tissue blocks containing the hypothalamus or hindbrain were dissected as described previously (19) and snap frozen. The retroperitoneal fat was dissected and weighed as an indicator of adiposity. Tissue O-GlcNAc, GFAT, OGT, O-GlcNAcase, pSTAT3(Y705), pSTAT3(S727), STAT3, and SOCS3 were measured by Western blot.

Experiment 2: the effect of GlcN on HBP activity and leptin signaling in mice. The objective of this experiment was to test whether activation of the HBP independent of glucose flux would stimulate STAT3(Y705) phosphorylation. Because activation of the HBP causes insulin insensitivity (7), blood glucose was used as an index of activation of the HBP under the assumption that development of insulin insensitivity would increased blood glucose concentrations. Male mice were fitted with jugular catheters and allowed to recover from surgery for 2 days by which time they had returned to their presurgical weight (30.4 ± 0.9 g presurgery; 30.2 ± 0.9 g postsurgery). On the test day they were moved to a procedure room and the catheter was attached to an infusion pump. Tail blood glucose was measured.

Table 2. Body weight and serum parameters in male mice offered chow or chow and 30% sucrose solution in the second part of experiment 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chow</th>
<th>Sucrose</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>26.7 ± 0.6</td>
<td>29.2 ± 1.9</td>
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<tr>
<td>Retropertitoneal fat, mg</td>
<td>187 ± 25</td>
<td>290 ± 32*</td>
</tr>
<tr>
<td>Serum measures</td>
<td></td>
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<tr>
<td>Glucose, mg/dl</td>
<td>168 ± 14</td>
<td>233 ± 57</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.63 ± 0.19</td>
<td>0.72 ± 0.11</td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>16 ± 4</td>
<td>17 ± 5</td>
</tr>
</tbody>
</table>

Data are means ± SE for groups of 6 mice. Measurements were made after the mice had been offered chow and water (Chow) or chow, water, and 30% sucrose solution (Sucrose) for 34 days. Mice were food deprived for 5 h before euthanasia. *P < 0.05, significant difference between groups.
measured at 10-min intervals until three stable readings had been obtained, and then the mice were infused at a rate of 1.2 μl/min with either saline or 0.3 μmol GlcN·10 g−1·min−1. At 90 min half of the mice in each treatment group received an intraperitoneal injection of saline and the other half received 2 mg leptin/kg (n = 5 or 6 per treatment group). The mice were killed at 120 min, tissues were collected and analyzed by Western blot as described for experiment 1, and serum insulin and leptin were measured.

Experiment 3: the effect of glucose, GlcN, leptin, and glucose plus leptin on HBP activity and leptin signaling in HepG2 cells. The objective of this experiment was to test whether the effects of drinking sucrose solution in mice could be replicated by exposing cells to high glucose concentrations. This would confirm that changes in leptin signaling were due to a direct effect of glucose on cell metabolism, rather than weight gain or a nonspecific, secondary response to consumption of sucrose solution. Human hepatoma HepG2 established cells (15-yr-old adolescent, Caucasian male; ATCC, Manassas, VA) were cultured in DMEM (Sigma, St. Louis, MO) and supplemented with 10% FBS (Sigma), 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), and 0.25 μg/ml fungizone antimycotic (Life Technologies). Cells were incubated in a humidified atmosphere of 5% CO2 at 37°C, cultured to passages 6 – 8, split from 90% confluent T-75 flasks to tissue culture plates, and grown to 75% confluence before treatment.

Cells were treated for 18 h in DMEM-low glucose (5.5 mM glucose; Sigma) or DMEM-low glucose modified with the addition of D-glucose (Sigma) or GlcN (Sigma) and supplemented as described above. The expression of pSTAT3(Y705), pSTAT3(S727), STAT3, SOCS3, in addition to O-GlcNAc, GFAT, OGT, and O-GlcNAcase was measured.

These initial studies identified 20 mM glucose as a concentration that stimulated, but did not maximize, activity of HBP and STAT3(Y705) phosphorylation. A 15-min exposure to 50 ng/ml leptin was the lowest dose of leptin that significantly increased HBP proteins and pSTAT3(Y705) compared with control. To test the interaction between activation of the HBP and leptin signaling cells were incubated for 18 h with 5.5 mM [low glucose (LG)] or 20 mM [high glucose (HG)] glucose and then treated with 0 or 500 ng leptin/ml (recombinant rat leptin; R&D Systems) for 15 or 30 min. The expression of pSTAT3(Y705), STAT3, SOCS3, GFAT, OGT, and O-GlcNAcase was measured.

The optimal concentration of glucose for stimulation of the HBP was determined by measuring O-GlcNAc protein modification, GFAT, OGT, and O-GlcNAcase in cells incubated for 18 h with 5.5 to 25 mM glucose. There were seven or eight replicates for each treatment condition. pSTAT3(Y705), STAT3, pERK1/2, pPI3K, and SOCS3 were also measured. Specificity of response was tested by incubating cells with 0.1 to 2.0 mM GlcN in the presence of 5.5 mM glucose. GlcN enters the HBP independent of GFAT and is a positive control for activation of the HBP by glucose. Leptin responsiveness of cells was tested by incubating cells with 5.5 mM glucose for 18 h and then treating them with 5 to 500 ng leptin/ml (recombinant rat leptin; R&D Systems) for 15 or 30 min. The expression of pSTAT3(Y705), STAT3, SOCS3, in addition to O-GlcNAc, GFAT, OGT, and O-GlcNAcase was measured.

Data are means ± SE for groups of 5 mice. *Significant differences between chow and sucrose mice from experiment 1. Data are means ± SE for groups of 5 mice. *Significant differences between chow and sucrose mice. Images have been adjusted for brightness and contrast.

Fig. 3. Hypothalamic STAT3 phosphorylation (A and B), total STAT3 (C), SOCS3 (D), O-GlcNAc protein modification (E), and HBP enzyme expression (F–H) measured in the second set of chow and sucrose mice from experiment 1. Data are means ± SE for groups of 5 mice. *Significant differences between chow and sucrose mice. Images have been adjusted for brightness and contrast.
To determine whether the changes in leptin signaling of HG cells were due to activation of the HBP, cells were incubated for 18 h with LG, HG, or LG + 1 mM GlcN in the presence or absence of 10 μM azaesarine. Azaesarine is a non-specific amido transferase inhibitor that downregulates GFAT, which is the rate-limiting enzyme for glucose entry into the HBP. Half of the LG cells were exposed to 50 ng leptin/ml for 15 min before harvest. The expression of pSTAT3(Y705), pSTAT3(S727), STAT3, SOCS3, O-GlcNAc, GFAT, OGT, and O-GlcNAcase was measured.

Western blotting. Protein samples were separated on 10% acrylamide gels and transferred to PVDF membrane. Membranes were blocked for 1 h with 5% dry milk or 1% BSA and exposed to primary antibody (Table 1) overnight at 4°C. Membranes were washed with Tris-0.1% Tween and Tris buffers, incubated with goat horseradish peroxidase anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) or goat horseradish peroxidase anti-mouse antibody (Sigma) for 1 h at room temperature, washed, exposed to ECL reagent (GE Healthcare Amersham, Little Chalfont, UK), and then exposed to X-ray film or imaged using the FOTOODYNE Luminary FX workstation (Hartland, WI). Membranes were stripped and reprobed for actin or total protein in the case of phosphorylated proteins (Table 1). Protein expression was quantified using Totallab Quant software (FOTOODYNE) and expressed as a ratio to control protein. Due to the variability among groups of cells, O-GlcNAc data for each treatment were expressed as fold change from control.

Statistical analysis. Significant differences (P < 0.05) between treatment groups were determined using Statistica software (StatSoft Version 9.0; Tulsa, OK). Data from animal studies were compared using repeated-measures ANOVA and post hoc t-tests or Tukey-Kramer test. Cell data for dose-response studies were analyzed by repeated-measures ANOVA and leptin response by two-way ANOVA followed by post hoc Tukey-Kramer’s test.

RESULTS

Experiment 1: effect of consumption of sucrose solution on leptin and insulin response in mice. Mice offered 30% sucrose solution consumed significantly more energy than their controls (Fig. 1A). The two groups of mice weighed the same at the start of the study (21 ± 1 g), and weights were not significantly different at the end (chow: 25 ± 1; sucrose 27 ± 1). Fat pads were larger in sucrose than chow mice, and this difference was significant for epididymal and retroperitoneal fat (Fig. 1B). Total dissected fat was increased by 60% (chow: 1,431 ± 156 mg; sucrose: 2,263 ± 320 mg). The sucrose mice were leptin and insulin insensitive (Fig. 1, C and D). At the end of the study there were no differences in serum glucose or insulin, but leptin was increased almost fourfold in sucrose mice (Fig. 1, E–G). Liver O-GlcNAc-modified protein and GFAT were increased and O-GlcNAcase decreased in sucrose mice (Fig. 2, A, B, and D). Sucrose consumption substantially increased basal pSTAT3(Y705) but not pSTAT3(S727) and inhibited SOCS3 expression (Fig. 2, E, F, and H). Total STAT3 did not change.

There were no differences in body weight, serum glucose, insulin, or leptin of the second set of chow and sucrose mice (Table 2). Retroperitoneal fat was heavier in the sucrose mice. Western blot analysis showed that pSTAT3(Y705) and O-GlcNAc proteins were significantly increased whereas pSTAT3(S727) was significantly decreased in hypothalamic tissue of sucrose mice compared with chow controls (Fig. 3, A, B, and E). There were no significant differences in hypothalamic total STAT3, SOCS3, GFAT, O-GlcNAcase, or OGT protein expression (Fig. 3, C, D, F, G, and H). Protein expression in the brainstem showed the same pattern of response as in the hypothalamus, but none of the differences reached statistical significance (Fig. 4). Analysis of the liver provided identical results as those found in the first set of animals. O-GlcNAc protein, GFAT, and pSTAT3(Y705) were all significantly increased in sucrose mice whereas O-GlcNAcase was significantly decreased (data not shown).

Experiment 2: the effect of GlcN on HBP activity and leptin signaling in mice. Blood glucose gradually declined during the 3-h infusion in saline-infused mice, but this was prevented in GlcN-infused mice. Leptin injection at 90 min did not have any significant effect on glucose; therefore, the blood glucose data in Fig. 5A are for both PBS and leptin-injected mice. There was no effect of GlcN or leptin on serum insulin measured at the end of the experiment (Fig. 5B). There was an exaggerated increase in serum leptin in the leptin-injected GlcN-infused mice (Fig. 5C). The acute GlcN infusion had no significant effect on protein expression in liver tissue (data not shown). By contrast, both leptin and GlcN increased hypothalamic O-GlcNAc (Fig. 5D), but there was no additive effect and we did not detect differences in GFAT, OGT, or O-GlcNAcase (Fig. 5, E–G). Both leptin and GlcN stimulated hypothalamic pSTAT3(Y705), with no additive effect. GlcN prevented a leptin-dependent stimulation of pSTAT3(S727) (Fig. 5, H and I).
HYPOTHALAMIC PROTEIN EXPRESSION – EXPERIMENT 2

**A** Blood Glucose

- **Saline**
- **GlcN**

Blood glucose (mg/dL) measured during a 3-h infusion of saline or glucosamine (GlcN) in experiment 2 (n = 10 or 11 mice). B and C: serum insulin and leptin measured at the end of the infusion. Hypothalamic O-GlcNAc protein (D), GFAT (E), β-N-acetylglucosaminidase (O-GlcNAcase; F), OGT (G), pSTAT3(Y705) (H), pSTAT3(S727) (I), and total STAT3 (J) measured in a block of hypothalamic tissue collected at the end of the experiment (n = 5 or 6 mice). Mice were injected intraperitoneally with saline or leptin 90 min before the end of the experiment, therefore, there were 4 treatment groups: saline/saline (SS), saline/leptin (SL), GlcN/saline (GS), and GlcN/leptin (GL). Data are means ± SE. Values for a specific protein that do not share a common superscript are significantly different. *Significant differences in blood glucose of saline- and GlcN-infused mice. Images are representative blots and have been adjusted for brightness and contrast.

Fig. 5. Blood glucose (A) measured during a 3-h infusion of saline or glucosamine (GlcN) in experiment 2 (n = 10 or 11 mice). B and C: serum insulin and leptin measured at the end of the infusion. Hypothalamic O-GlcNAc protein (D), GFAT (E), β-N-acetylglucosaminidase (O-GlcNAcase; F), OGT (G), pSTAT3(Y705) (H), pSTAT3(S727) (I), and total STAT3 (J) measured in a block of hypothalamic tissue collected at the end of the experiment (n = 5 or 6 mice). Mice were injected intraperitoneally with saline or leptin 90 min before the end of the experiment, therefore, there were 4 treatment groups: saline/saline (SS), saline/leptin (SL), GlcN/saline (GS), and GlcN/leptin (GL). Data are means ± SE. Values for a specific protein that do not share a common superscript are significantly different. *Significant differences in blood glucose of saline- and GlcN-infused mice. Images are representative blots and have been adjusted for brightness and contrast.
and there were no differences in total STAT3 (Fig. 5J) or SOCS3 expression (data not shown).

**Experiment 3:** the effect of glucose, GlcN, leptin, and glucose plus leptin on HBP activity and leptin signaling in HepG2 cells. O-GlcNAc protein levels and GFAT expression in HepG2 cells treated with 20 or 25 mM glucose were significantly increased compared with 5.5 mM control cells (Fig. 6, A and B). pSTAT3(Y705) was increased by 56% in cells incubated with 20 mM glucose and by 72% in cells exposed to 25 mM glucose compared with control (Fig. 6C). Glucose concentration had no effect on OGT, O-GlcNAcase, total STAT3, or SOCS3 (data not shown). Concentrations of 0.5 mM or higher GlcN increased O-GlcNAc protein compared with control conditions, and pSTAT3(Y705) was increased in cells treated with 1.0 or 1.5 mM GlcN (Fig. 7, A and B). GlcN had no effect on pSTAT3(S727) (Fig. 7C), SOCS3 (Fig. 7D), total STAT3, GFAT, OGT, or O-GlcNAcase (data not shown). Exposure to 10 ng/ml leptin for 15 min produced a significant increase in pSTAT3(Y705) (Fig. 8A), whereas 5 ng/ml stimulated GFAT expression (Fig. 8B). There was a significant increase in O-GlcNAc protein in cells exposed to 50 or 100 ng leptin/ml for 15 min (Fig. 8C). There were no significant differences in STAT3, SOCS3, OGT, or O-GlcNAcase at 15 min and no difference in expression of any protein at 30 min (data not shown).

LG + leptin and HG each produced similar increases in O-GlcNAc protein and GFAT expression, but there was no additive effect (Fig. 9, A and B). Neither condition produced a change in OGT or O-GlcNAcase expression (data not shown). Similarly, LG + leptin and HG stimulated pSTAT3(Y705), with no additive effect (Fig. 9C). LG + leptin also stimulated pSTAT3(S727), whereas there was no effect of HG or HG + leptin (Fig. 9D). Neither HG nor leptin had any effect on pPI3K, pERK1/2, total STAT3, or SOCS3 expression (data not shown).

The stimulatory effects of leptin, HG, and GlcN on O-GlcNAc protein were confirmed in the control cells for the azaserine experiment. Although azaserine significantly inhibited GFAT expression in LG + leptin and HG cells, it did not inhibit total O-GlcNAc protein modification (Fig. 10, A and B). OGT expression was increased by azaserine in all cells, except those incubated with GlcN (Fig. 10C). O-GlcNAcase was stimulated by azaserine in LG cells, but there was no effect with other treatments where there was already a tendency for O-GlcNAcase to be stimulated in control conditions (Fig. 10D). Leptin, HG, and GlcN all stimulated pSTAT3(Y705) in control conditions, and this was prevented by azaserine in LG + leptin and HG cells but not GlcN cells (Fig. 10E). pSTAT3(S727) was stimulated by LG + leptin, and this was partially reversed by azaserine (Fig. 10F). Azaserine also increased SOCS3 expression, but this reached significance only in LG cells (Fig. 10G).

**DISCUSSION**

Previously, we have shown that rats offered chow, lard, and sucrose solution chow and sucrose solution become resistant to the effects of leptin on food intake more quickly than rats fed a high-fat diet (13) and that 2 days of continuous GlcN infusion also makes rats resistant to the hypophagic effects of leptin (41), supporting the idea that activation of the HBP and the resulting O-GlcNAc protein modification cause leptin resistance. The objective of the studies described here was to test whether leptin resistance in sucrose-drinking mice was associated with increased activity of the HBP and to use an in vitro system to examine the direct effect of glucose on HBP activity and leptin signaling. The animal studies confirm that consumption of 30% sucrose solution increases O-GlcNAc-modification of proteins and results in insulin insensitivity and resistance to the effect of leptin on food intake. The in vitro

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![GLUCOSE DOSE RESPONSE](Image)

**Fig. 6.** Protein expression in HepG2 cells treated with increasing concentrations of glucose for 18 h. Total O-GlcNAc protein (A), GFAT (B), and pSTAT3(Y705) (C) were all increased by higher glucose concentrations. Data are means ± SE for 7 or 8 replicates. Values that do not share a common superscript are significantly different. Images are representative blots and have been adjusted for brightness and contrast.
studies show that increased glucose and leptin can each independently increase O-GlcNAcylation of proteins, GFAT and pSTAT3(Y705) but that there is no additive effect. Unexpectedly, we found that glucose inhibited leptin-induced phosphorylation of STAT3(S727).

As noted above, experiment 1 confirmed that access to a 30% sucrose solution increased protein O-GlcNAcylation in both liver and hypothalamic tissue. Western blot data indicated that increased protein O-GlcNAc modification in the liver was associated with an increase in GFAT expression, which would facilitate glucose entry into the pathway (30), and by decreased O-GlcNAcase expression, which would reduce the rate of removal of O-GlcNAc from modified proteins (8). There were no significant changes in protein levels of HBP enzymes in the hypothalamus, although there was a trend for an increase in OGT and decrease in O-GlcNAcase. The resulting change in the ratio of these two enzymes would potentially increase protein O-GlcNAc modification. The absence of significant changes in activation of leptin signaling proteins or HBP enzymes in the brainstem of sucrose mice may be due to the low level of expression of ObRb in this area compared with the hypothalamus or because we collected tissue after the mice had been food deprived for 5 h. If we had collected tissue from fed sucrose mice, it is possible that we would have found an increase in HBP activity.

There is a large literature supporting a contribution from chronic activation of the HBP towards the development of whole animal insulin resistance and inhibition of glucose uptake by muscle and adipose tissue (46). Although the mechanism has not yet been elucidated (7), it is clear that increased glucose or GlcN flux through the pathway results in O-GlcNAcylation of the insulin receptor and insulin signaling proteins (44, 48, 49). Proteins that are common to insulin and leptin signaling, including IRS1, PI3K, and STAT3, are subject to O-GlcNAcylation (37, 49). Therefore, it was reasonable to test whether increased activity of the HBP induces leptin resistance. The sucrose mice were resistant to the effects of leptin on food intake when they were tested between days 33 and 38, which is comparable to the time at which rats offered sucrose solution showed a similar resistance to peripheral leptin injections (13). The leptin resistance was accompanied by a fourfold increase in serum leptin concentration even though body fat was increased by only 60%, consistent with reports that increased HBP activity stimulates leptin expression and release (43). We did not find a comparable increase in serum leptin in the GlcN-infused mice even though they
showed the same changes in STAT3 activation as the sucrose mice. Previously, we found that rats infused with GlcN for 2 days did not respond to leptin’s effect on food intake (41), but they were not hyperleptinemic (unpublished data); therefore, it is unlikely that the failure of GlcN to induce leptin in experiment 2 was due to the short duration of the GlcN infusion. We did not investigate adipose tissue glucose metabolism or measure leptin mRNA expression in any study, but sucrose mice were fatter than their controls, whereas GlcN-infused animals were not and it is possible that the increase in basal leptin expression is dependent on a combination of increased fat mass and increased glucose availability.

The changes in HBP activity and activation of leptin signaling protein in livers of sucrose drinking mice in experiment 1 also were not replicated in GlcN-infused mice in experiment 2. We previously reported that 2 days of continuous GlcN infusion caused leptin resistance and increased hypothalamic and brainstem pSTAT3(Y705), even though GlcN did not increase liver total O-GlcNAc protein or HBP enzyme levels (41); therefore, it is possible that activation of the hepatic HBP independent of GFAT activity minimizes the effect of substrate flux on pathway enzyme expression. O-GlcNAc has been reported to inhibit liver glucokinase (4, 2), which would limit glucose uptake and activation of the HBP in both sucrose and GlcN-infused mice and is unlikely to explain the differences between sucrose and GlcN-infused mice in our studies. By contrast, glucokinase activity is stimulated by fructose-1-phosphate (42), and this could have contributed to a difference in hepatic glucose handling by sucrose-drinking and GlcN-infused mice. Even though we did not find any effect of acute GlcN infusion on liver protein expression, we did find an association between HBP activity and changes in phosphorylation state and leptin responsiveness of STAT3 in the hypothalamus. These data are indicative of changes in central as well as peripheral leptin signaling and confirm that peripheral administration of HBP substrate has the potential to modify carbohydrate metabolism in brain tissue. In the studies described here we showed that sucrose mice were resistant to the hypophagic effect of leptin and that hypothalamic pSTAT3(Y705) was elevated in basal conditions. Although factors other than leptin stimulate pSTAT3 (10) and we did not confirm that the pSTAT3 was increased specifically in cells that express ObRb, it is likely that there was colocalization for at least some of the cells and we have previously reported that leptin does not stimulate pSTAT3 above basal rates in sucrose-drinking rats (15). Similarly, we show that leptin does not stimulate already high levels of pSTAT3(Y705) in GlcN-infused mice. Although we did not measure a behavioral response to leptin in these mice, we have previously reported that rats infused with GlcN for 2 days are resistant to the effect of leptin on food intake (41).

The increase in basal pSTAT3(Y705) and inhibition of leptin-induced pSTAT3(S727) in cells incubated with 20 mM glucose indicates that leptin resistance in mice and rats (13) consuming sucrose solution is a direct response to increased glucose metabolism, rather than obesity or a nonspecific effect of sucrose consumption. HepG2 cells are not phenotypic of brain or liver cells, but there is no cell line, including secretory hypothalamic GT1–7 cells (31), that can replicate the hypothalamic metabolism that is controlled by neural afferents, glia, and extracellular metabolite concentration. While it is possible that some of the biochemical responses examined in HepG2 cells in the current study are not representative of changes in vivo, HepG2 cells do express a functional HBP (17) and ObRb (54) and have been used to examine the effects of fructose on cell fatty acid and glucose metabolism, including UDP-GlcNAc production (17). Incubating the cells in HG media increased O-GlcNAc modification of proteins and GFAT expression, similar to the changes observed in liver and hypothalamic tissue from sucrose mice and confirming a previous report that pSTAT3(Y705) levels are increased in HepG2 cells incubated with 25 mM glucose for 12 h (24). Therefore,
HepG2 cells provide a useful in vitro model for investigating the mechanism by which nutrient activation of the HBP directly affects leptin signaling even though they are unlikely to perfectly replicate any specific tissue taken from mice consuming sucrose solution.

Although infusion of GlcN for 3 h increased hypothalamic protein O-GlcNAcylation, there were no measurable changes in OGT or O-GlcNAcase that could account for the increased rate of protein modification. A similar response was found in HepG2 cells exposed to GlcN for 18 h. Multiple splice variants of OGT have been identified (16), and each isoform performs a unique intracellular function (26). Similarly, O-GlcNAcase has two isoforms, but the long form accounts for a majority of the activity (20). Future studies will use more specific antibodies to determine whether there are changes in expression of a specific isoform of one or both of the enzymes. Glucose could also potentially alter OGT and O-GlcNAcase activities without a detectable change in enzyme protein levels. Nevertheless, increased protein O-GlcNAcylation confirms that GlcN produced the expected activation of the HBP and there was a simultaneous change in STAT3 phosphorylation identical to that caused not only by glucose activation of the HBP in HepG2 cells but also in liver and hypothalamus of sucrose mice and hypothalamus of GlcN-infused mice.

An unexpected finding was that addition of leptin to LG media produced similar increases in O-GlcNAc, GFAT, and pSTAT3(Y705) as those that were caused by 18-h exposure to HG media, but there was no additive effect of leptin on the HG response. To our knowledge, these are the first in vivo or in vitro studies to demonstrate that leptin controls HBP activity and further investigation is needed to elucidate the physiologic and metabolic consequences of this control system. The stimulatory effect of leptin on both HBP activity and phosphorylation of STAT3(Y705) was significant within 15 min of treatment but had declined by 30 min. This short-lived activity of leptin has been reported by others for different in vitro cell culture models (18, 34) and is similar to the rapid decline in hypothalamic pSTAT3 produced by a leptin injection in rats (53). Inhibition of GFAT with azaserine did not suppress total O-GlcNAc protein or GlcN-induced pSTAT3(Y705) but did prevent HG and leptin-induced pSTAT3(Y705) and also increased SOCS3. As expected, GlcN-induced activation of pSTAT3(Y705) was not significantly inhibited by azaserine because GlcN enters the HBP independent of GFAT. Both inhibition of glucose entry into the HBP and increased expression of SOCS3 could have contributed to the downregulation of pSTAT3(Y705); however, because azaserine stimulated SOCS3 in the GlcN cells, but there was little change in

Fig. 9. Protein expression in HepG2 cells incubated with 5.5 mM glucose (LG) or 20 mM glucose (HG) for 18 h and either PBS (LG and HG) or 50 ng leptin/ml (LG + L, HG + L) for 15 min. Leptin and HG stimulated O-GlcNAc protein (A), GFAT (B), and pSTAT3(Y705) (C). Leptin stimulated pSTAT3(S727) in LG but not HG cells (D). Data are means ± SE for 7 or 8 replicates. Values that do not share a common superscript are significantly different. Images are representative blots and have been adjusted for brightness and contrast.
pSTAT3(Y705), this implies that inhibition of the HBP was the primary cause of downregulation of pSTAT3(Y705) in LG + leptin and HG cells. One reason we did not find a change in total O-GlcNAc protein in azaserine-treated cells is likely due to the increase in OGT expression without a compensatory increase in O-GlcNAcase. The increase in OGT expression may be an indirect measure of effective downregulation of glucose entry into the HBP because OGT shows auto-GlcNAcylation (23) and glucose starvation increases OGT protein expression (6).

STAT3 phosphorylation is essential for the control of both food intake and energy expenditure by leptin (3). Disruption of normal leptin signaling due to increased flux into the HBP represents a state of leptin resistance in which basal pSTAT3(Y705) is increased to a level that is not different from normal leptin stimulated levels and cannot be further increased by leptin. Many animals made leptin resistant by extended exposure to high-fat diet (21, 50) or aging (38) show a small increase in basal pSTAT3(Y705) and a failure of leptin to phosphorylate STAT3 to the same level as found in leptin-responsive animals (33). By contrast, others have reported that basal pSTAT3(Y705) immunoreactivity in leptin-resistant high-fat fed mice is similar to that in leptin-treated mice on a low-fat diet with no additive effect of leptin treatment (22, 28). These results are similar to those reported here and imply a complex relationship among glucose metabolism, leptin production, and leptin activity because it has previously been established that increased HBP activity promotes adipocyte leptin expression and release (9, 43).

The data described here suggest that leptin resistance is associated with increased basal levels of pSTAT3(Y705) but a failure to phosphorylate STAT3(S727). Others have reported that STAT3 can be O-GlcNAc modified (11, 49), but the sites that are modified have not been mapped and the impact on STAT3 function has not been determined. Because O-GlcNAc modification occurs on serine and threonine residues of proteins, S727 is a potential target for O-GlcNAcylation, which may explain why phosphorylation was inhibited. Taylor et al (39) reported that both elevation and depletion of media glucose can O-GlcNAc-modify different proteins, which results in changes in metabolism that are appropriate for substrate availability. Therefore, although we found an overall increase in

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**Fig. 10.** Protein expression in HepG2 cells incubated with 5.5 mM glucose (LG), 20 mM glucose (HG), or LG + 1 mM GlcN for 18 h with or without 10 μM azaserine. Half of the LG cells were then incubated 50 ng leptin/ml (LG + L) for 15 min. Azaserine increased O-GlcNAc protein in LG cells (A) and inhibited leptin and HG stimulation of GFAT (B). Azaserine increased OGT (C) but not O-GlcNAcase (D) expression in LG- and HG-treated cells. Azaserine prevents the stimulation of pSTAT3(Y705) by leptin and HG but not GlcN treatment (E). There was no effect on pSTAT3(S727) (F), but SOCS3 was stimulated in azaserine-treated LG cells (G). Data are means ± SE for 7 or 8 replicates. Values that do not share a common superscript are significantly different.
O-GlcNAc-protein, it is possible that some proteins were O-GlcNAc modified in low glucose, but not in high glucose conditions. We did not determine whether the HBP-induced increase in pSTAT3(Y705) was a due to a direct effect on STAT3 or whether the exaggerated phosphorylation was secondary to activation of ObRb. Sites other than Ser727 on STAT3 may also have been modified and accounted for increased phosphorylation of Y705. Although it has been shown that activation of STAT3(Y705) is essential for effective leptin signaling, little is known about the role of pSTAT3(S727) and additional studies are needed to determine whether glucose-induced pSTAT3(Y705) is bioactive in the absence of pSTAT3(S727). The simultaneous activation of the HBP and STAT3 following leptin treatment suggests that this is a mechanism that could modify leptin action, either enhancing the response by increasing pSTAT3(Y705) or downregulating the response by inhibiting pSTAT3(S727). Further studies are needed to elucidate the biological relevance of the acute leptin-induced stimulation of the HBP.

In summary, our results suggest that under conditions of sustained increases in HBP activity there is an increase in basal STAT3(Y705) phosphorylation that cannot be further increased by leptin. We have previously shown that activation of the HBP by GlcN infusion makes rats resistant to leptin-induced hypophagia (41), and others have reported increased basal pSTAT3(Y705) levels in leptin-resistant animals fed a high-fat diet (22, 28). These latter studies, however, did not measure activation of the HBP. Although pSTAT3(Y705) is increased, high glucose appears to inhibit phosphorylation of STAT3(S727), which normally enhances pSTAT3 transcriptional activity (34), raising the possibility that HG-induced pSTAT3(Y705) does not have the same bioactivity as leptin-induced pSTAT3(Y705). Furthermore, we provide new evidence that in low glucose conditions leptin directly activates the HBP and increases O-GlcNAcylation of proteins. Additional studies are needed to elucidate both how activation of the HBP affects leptin signaling and the implications of control of HBP activity by leptin.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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