Chronic Hexosamine Flux Stimulates Fatty Acid Oxidation by Activating AMP-activated Protein Kinase in Adipocytes

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The hexosamine biosynthesis pathway (HBP) serves as a nutrient sensor and has been implicated in the development of type 2 diabetes. We previously demonstrated that fatty acid oxidation was enhanced in transgenic mouse adipocytes, wherein the rate-limiting enzyme of the HBP, glutamine:fructose-6-phosphate amidotransferase (GFA), was overexpressed. To explore the molecular mechanism of the HBP-induced fatty acid oxidation in adipocytes, we studied AMP-activated protein kinase (AMPK), an energy sensor that stimulates fatty acid oxidation by regulating acetyl-CoA carboxylase (ACC) activity. Phosphorylation and activity of AMPK were increased in transgenic fat pads and in 3T3L1 adipocytes treated with glucoseamine to stimulate hexosamine flux. Glucosamine also stimulated phosphorylation of ACC and fatty acid oxidation in 3T3L1 adipocytes, and these stimulatory effects were diminished by adenovirus-mediated expression of a dominant negative AMPK in 3T3L1 adipocytes. Conversely, blocking the HBP with a GFA inhibitor reduced AMPK activity, ACC phosphorylation, and fatty acid oxidation. These changes are not explained by alterations in the cellular AMP/ATP ratio. Further demonstrating that AMPK is regulated by the HBP, we found that AMPK was recognized by succinylated wheat germ agglutinin, which specifically binds O-GlcNAc. The levels of AMPK in succinylated wheat germ agglutinin precipitates correlated with hexosamine flux in mouse fat pads and 3T3L1 adipocytes. Moreover, removal of O-GlcNAc by hexosaminidase reduced AMPK activity. We conclude that chronically high hexosamine flux stimulates fatty acid oxidation by activating AMPK in adipocytes, in part through O-linked glycosylation.

Although there is a major genetic contribution to type 2 diabetes, the largest predisposing factor remains caloric excess and/or obesity. Underlining the importance of this mechanism, excess glucose and lipids themselves can cause the pathological hallmarks of diabetes, insulin resistance, and β-cell failure. One pathway by which excess nutrients can contribute to the diabetic phenotype is the hexosamine biosynthesis pathway (HBP) (1–4). In this pathway, a relatively small amount of cellular glucose flux is converted to UDP-GlcNAc and other amino sugars. The rate-limiting step is catalyzed by enzyme glutamine:fructose-6-phosphate amidotransferase (GFA), and the levels of the product UDP-GlcNAc are proportional to cellular glucose flux and thus able to serve a nutrient sensing function. In the short run, hexosamines function as physiologic glucose sensors that serve an adaptive role in directing excess calories toward storage as fat. When chronically stimulated, however, the HBP can also lead to insulin resistance, hyperinsulinemia, hyperlipidemia, and hyperleptinemia (2,3,5–7).

UDP-GlcNAc, the chief product of the pathway, is the substrate for O-glycosyltransferase, which catalyzes the O-linked glycosylation of nuclear and cytosolic proteins with a single N-acetylgalactosamine (O-GlcNAc) moiety on serine and threonine residues (8–11). It has been demonstrated that hexosamines regulate metabolism through this mechanism of O-linked protein glycosylation (12,13). For example, high glucose flux through the hexosamine pathway induces O-GlcNAc modification of glycogen synthase and inhibits its enzymatic activity in streptozotocin diabetic mice and cultured 3T3L1 adipocytes (14,15).

AMP-activated protein kinase (AMPK) plays a pivotal role in regulating energy and metabolic homeostasis and has been dubbed a cellular energy fuel gauge (16–19). AMPK is activated by phosphorylation of Thr172 in a number of physiologic and pathologic states, wherein an increase in the AMP/ATP ratio occurs in the cell (16,19,20). Once activated, AMPK phosphorylates downstream targets to inhibit ATP-utilizing pathways and stimulate ATP-generating pathways (17,18). One of the best characterized mechanisms of AMPK in metabolic regulation is its induction of fatty acid oxidation; activation of AMPK stimulates the phosphorylation of acetyl-CoA carboxylase (ACC), which leads to the inhibition of ACC activity and a consequent reduction in malonyl-CoA content, thereby allowing fatty acid uptake into mitochondria and subsequent oxidation (21,22). We have previously reported that transgenic mouse adipocytes overexpressing GFA at high but physiologic levels exhibited enhanced fatty acid oxidation (23). This enhanced fat
oxidation is somewhat paradoxical, because a pathway that signals fuel excess would be expected to preferentially stimulate fat synthesis. On the other hand, relaxation of a prohibition on fat oxidation in condition of chronic fuel excess would probably be adaptive. In the present study, we demonstrate that hexosamine flux up-regulates AMPK activity in differentiated 3T3L1 adipocytes and transgenic mouse fat pads, resulting in increased phosphorylation of ACC and activation of fatty acid oxidation. We also found that increased AMPK activity was associated with increased levels of O-GlcNAc protein modification on AMPK or a binding partner and was reversed by enzymatic removal of O-GlcNAc. These findings demonstrate a direct link between two important nutrient sensing pathways, the HBP and AMPK.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phosphatase inhibitor mixture, diazooxonorleucine (DON), glucosamine, the recombinant catalytic subunit of protein phosphatase 1α, and β-N-acetylglucosaminidase were obtained from Sigma. Dulbecco’s modified Eagle’s medium (DMEM) containing high glucose (20 mM) and all other cell culture reagents were obtained from Invitrogen. [9,10-3H]Palmitic acid (10 mCi/ml) and [γ-32P]ATP (25 Ci/mmol) were purchased from MP Biomedicals. The protease inhibitors were purchased from Roche Applied Science. Antibodies to phosho-AMPKα (Thr172) and AMPKα were purchased from Cell Signaling Technology (Beverly, MA). AMPKα1 and phosho-ACC (Ser79) antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-O-GlcNAc monoclonal IgM antibody (CTD 110.6) is a gift from Dr. Gerald Hart (Johns Hopkins University). Succinylated wheat germ agglutinin-agarose (sWGA) was obtained from EY Laboratories (San Mateo, CA).

**Transgenic Animals**—Transgenic mice (aP2-GFA) wherein GFA was overexpressed specifically in adipose tissue under control of the aP2 promoter have been described previously (7). In brief, epididymal fat pads from wild type and transgenic mice were collected after sacrifice and immediately mixed with an equal volume of radioimmune precipitation supernatant were used for immunoprecipitation. Cell or tissue lysates were precleared with normal rabbit IgG together with protein A/G-agarose (Santa Cruz Biotechnology) for 30 min at 4 °C, and then the supernatants were incubated with anti-AMPKα1 for 2 h at 4 °C. The immunocomplexes were collected using protein A/G-agarose, washed three times with lysis buffer and once with AMPK kinase reaction buffer (40 mM HEPES, 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 0.8 mM dithiothreitol, 5 mM MgCl2, 0.2 mM ATP, 0.2 mM AMP). AMPK immunoprecipitates were incubated with 20 μl of AMPK kinase reaction buffer, 5 μl of SAMS substrate peptide (1 mM), and 2 μCi of [γ-32P]ATP for 30 min at 37 °C. At the end of the incubation, an aliquot was removed and spotted onto Whatman P81 paper. The P81 paper was washed three times with 0.75% (v/v) phosphoric acid and once with acetone. The paper was transferred to a vial containing 3 ml of scintillation mixture, and radioactivity was determined by scintillation counting.

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**Adenovirus-mediated Gene Transfer**—Replication-defective recombinant adenovirus was generated as described (24). AdEasy system components were provided by Dr. Bert Vogelstein (Johns Hopkins University). To generate the adenoviral vector expressing a dominant negative mutant of AMPKα2 (DN-AMPKα2), AMPKα2 cDNA bearing a mutation of lysine 45 to arginine (K45R) (a gift from Dr. Morris Birnbaum; University of Pennsylvania) was subcloned into a shuttle vector (pAdTrack-CMV). The pAd-DN-AMPK plasmid was linearized by digestion with PmlI and co-transformed into Escherichia coli BJ5183 with the adenoviral backbone plasmid, pAdEasy-1. Homologous recombinants were selected for kanamycin resistance. Finally, the linearized recombinant plasmid was transfected into transformed HEK293 cells. Recombinant adenoviruses were amplified in HEK293 cells. Differentiated 3T3L1 adipocytes were infected with the adenovirus for 2 days. After removal of virus-containing medium, cells were treated with GFA inhibitor (DON) or glucosamine in fresh culture medium for 24 h. A replication-defective virus expressing green fluorescence protein (GFP) was used as a control. Preliminary studies revealed that after 48 h of infection with control GFP, >80% of 3T3L1 adipocytes expressed green fluorescence protein.

**AMPK Assays**—Differentiated 3T3L1 adipocytes were harvested in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease inhibitors, phosphatase inhibitors), allowed to lyse for 20 min on ice, and then sonicated for 5 s, followed by centrifugation at 16,000 × g for 20 min to remove debris. Fat pad lysates were obtained as described previously (7). In brief, epididymal fat pads from wild type and transgenic mice were collected after sacrifice and immediately placed in ice-cold lysis buffer. After sonication for 5 s, the samples were centrifuged at 16,000 × g for 20 min at 4 °C, and the supernatant were used for immunoprecipitation. Cell or tissue lysates were precleared with normal rabbit IgG together with protein A/G-agarose (Santa Cruz Biotechnology) for 30 min at 4 °C, and then the supernatants were incubated with anti-AMPKα1 for 2 h at 4 °C. The immunocomplexes were collected using protein A/G-agarose, washed three times with lysis buffer and once with AMPK kinase reaction buffer (40 mM HEPES, 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 0.8 mM dithiothreitol, 5 mM MgCl2, 0.2 mM ATP, 0.2 mM AMP). AMPK immunoprecipitates were incubated with 20 μl of AMPK kinase reaction buffer, 5 μl of SAMS substrate peptide (1 mM), and 2 μCi of [γ-32P]ATP for 30 min at 37 °C. At the end of the incubation, an aliquot was removed and spotted onto Whatman P81 paper. The P81 paper was washed three times with 0.75% (v/v) phosphoric acid and once with acetone. The paper was transferred to a vial containing 3 ml of scintillation mixture, and radioactivity was determined by scintillation counting.

**Immobilization of AMPK with Wheat Germ Agglutinin and Western Blotting**—3T3L1 adipocyte or fat pad lysates were mixed with an equal volume of radioimmune precipitation buffer (11 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1% IGEPA, 0.1% SDS, 40 mM NaF, 0.5 mM 2-acetamido-1-aminoo-1,2-dideoxyglucopyranose, and protease inhibitors). The mix-
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ture was precleared with protein A/G-agarose for 30 min at 4 °C, and the supernatants were incubated with 50 μl of sWGA on a rotator for 16 h at 4 °C, followed by washing three times with radioimmune precipitation buffer/lysis buffer (1:1, v/v). sWGA precipitates were used for Western blotting. Western blotting was carried out as previously described (14). Densitometry measurements were obtained using NIH ImageJ software. In hexosaminidase digestion experiments, the sWGA precipitates were incubated with 8 units of β-N-acetylglucosaminidase from jack beans for 30 min at 30 °C before the AMPK activity assay (14, 15). In phosphatase digestion experiments, the 3T3L1 adipocytes were harvested in lysis buffer without EDTA and phosphatase inhibitors, and then the lysates were incubated with 7 units of protein phosphatase-1 (PP1a) for 30 min at 37 °C as described by Parker et al. (14).

Fatty Acid Oxidation Assay—Palmitic acid oxidation was assayed as described (23). 3T3L1 adipocytes in 60-mm dishes were cultured in 2 ml of DMEM containing 2% bovine serum albumin, 0.3 mM palmitic acid, and 20 μCi of [9,10-3H]palmitic acid. After 2 h, palmitic acid oxidation was assessed by measuring 3H2O produced in the incubation medium. The media (0.5 ml) were extracted by the addition of 2.5 ml of methanol/chloroform (1:2, v/v) and 1 ml of 2 x KCI/HCl (1:1, v/v), followed by centrifugation at 5,000 × g for 15 min. 3H2O release in the aqueous phase was measured by liquid scintillation counting. Background 3H2O release, as measured in an aliquot of medium with [9,10-3H]palmitic acid that was incubated without cells, was subtracted from experimental values.

Measurement of AMP, ADP, and ATP Content—Epididymal fat pads from both wild type and transgenic mice were lysed in ice-cold lysis buffer containing 5% (v/v) perchloric acid. After sonication for 5 s, the samples were centrifuged at 16,000 × g for 10 min at 4 °C twice to remove acid-insoluble material. Perchloric acid was extracted from the supernatant by three washes of tri-n-octylamine and 1,1,2-trichlorotrifluoroethane (1:1, v/v), followed by centrifugation at 14,000 × g for 2 min. To measure adenine nucleotide concentrations in 3T3L1 adipocytes, each well of 3T3L1 adipocytes in 6-well plates was harvested in 300 μl of lysis buffer containing 5% (v/v) perchloric acid. Adenine nucleotides were extracted as described above.

Nucleotides were separated by ion exchange chromatography on a Waters Novapak C18 column (3.9 × 150 mm). The column was equilibrated in Buffer A (35 mM potassium phosphate, 6 mM tetrabutylammoniumhydroxysulfate, 1.25 μM EDTA, pH 6.0) and developed with a linear gradient from 98% Buffer A, 2% Buffer B (Buffer A/acetonitrile, 1:1) to 50% Buffer A, 50% Buffer B over a 10-min period at a flow rate of 1.5 ml/min. Nucleotides were detected by their absorbance at 261 nm and compared with external standards.

Statistical Analysis—Descriptive statistics are represented as the means ± S.E. for the indicated number of experiments. Data were analyzed by Student’s t test (two-tailed) or by one-way analysis of variance and Student-Newman-Keuls test for post hoc analysis for multiple-group comparisons using GraphPad InStat software (San Diego, CA). Differences were considered significant when p < 0.05.

RESULTS

Overexpression of GFA in Fat Pads Stimulates AMPK Activity and Phosphorylation of AMPK and ACC—We have previously generated aP2-GFA transgenic mice that overexpress GFA specifically in adipose tissue and found that these transgenic adipocytes with increased hexosamine flux exhibited significantly higher rates of fatty acid oxidation (23). It is known that activation of AMPK stimulates fatty acid oxidation by phosphorylation and inhibition of ACC activity (18, 21). Therefore, we hypothesized that AMPK might mediate the effect of hexosamine flux on fatty acid oxidation in adipocytes. AMPK activity is responsive to feeding status as well as hormones, including leptin and adiponectin (17, 25, 26). Therefore, to minimize these effects and maintain carefully controlled hormonal and metabolic conditions, we fasted animals for 24 h and then collected the epididymal fat pads and examined AMPK activity. In adipose tissue, the α1 catalytic subunit is the predominant isoform expressed and accounts for the majority of AMPK activity in adipose tissue, the α1-containing AMPK immunoprecipitates increased to 153 ± 16% (p < 0.05) in fat pads from transgenic mice as compared with wild type littermates. We also observed increased total AMPK activity in ammonium sulfate precipitates of transgenic mouse fat pads compared with external standards. We also observed increased total AMPK activity in ammonium sulfate precipitates of transgenic mouse fat homogenates (data not shown). Previous studies have shown that phosphorylation of Thr172 within the AMPKα subunit is essential for its activation (31). Consistent with increased AMPK activity, overexpression of GFA in fat pads led to increased phosphorylation of AMPKα (159 ± 19%, p < 0.05; Fig. 1B). There was no significant difference in the levels of
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AMPKα protein and mRNA in transgenic and wild type fat pads (Fig. 1 B and data not shown). To further assess AMPK activity, we also determined the phosphorylation of its downstream target, ACC. Western blot analysis showed that transgenic fat pads had increased phosphorylation of ACC at Ser79 (205 ± 28%, p < 0.05), which paralleled the stimulation of AMPK activity and its Thr172 phosphorylation.

Chronic Flux through the HBP Increases AMPK Activity and Fatty Acid Oxidation in 3T3L1 Adipocytes—In the intact animal models, AMPK is under the influence of many intracellular and extracellular factors, including leptin and adiponectin (25, 26). To eliminate some of these influences, we examined the effect of increased hexosamine flux in 3T3L1 adipocytes, which do not exhibit changes in adiponectin and leptin levels with chronic hexosamine treatment (data not shown). To mimic chronically increased hexosamine flux, we treated differentiated 3T3L1 adipocytes with glucosamine for 24 h. Glucosamine enters adipocytes through the glucose transport system, is phosphorylated by hexokinase, and directly enters the hexosamine pathway downstream of GFA (3, 32). Consistent with our previous studies (14), overnight treatment of 3T3L1 adipocytes with glucosamine increased flux through the hexosamine pathway and significantly increased the levels of cellular O-linked GlcNAc on protein (Fig. 2 A). Treatment of cells with glucosamine (0.5–10 mM) increased the phosphorylation of AMPKα and ACC in a dose-dependent manner, with no change in expression of the AMPK α subunit. We further verified that glucosamine treatment also increased AMPKα1 activity measured in vitro (Fig. 2 B). Stimulation was observed at a glucosamine concentration of 0.5 mM and was nearly maximal at 2 mM. We next blocked the hexosamine biosynthesis pathway in 3T3L1 adipocytes by treating cells for 24 h with DON, a glucosamine analog that irreversibly inhibits GFA activity (3, 32). Blocking glucose flux through the HBP significantly inhibited AMPKα1 activity in 3T3L1 adipocytes (Fig. 2 C). DON treatment did not, however, affect glucosamine-induced AMPK activation. Because glucosamine enters the HBP downstream of GFA, the results with DON confirm that AMPK activation is mediated by the hexosamine biosynthesis pathway and is not due to nonspecific inhibitory effects of DON. The changes in AMPK activity are accompanied by the expected parallel changes in AMPK and ACC phosphorylation. These results demonstrate that chronic hexosamine flux through the HBP increase AMPK activity.

Activation of AMPK has been shown to stimulate fatty acid oxidation through the phosphorylation and inhibition of ACC (21). To confirm that the activation of AMPK by chronic hexosamine flux also resulted in this major downstream effect, we examined fatty acid oxidation in 3T3L1 adipocytes. Differentiated 3T3L1 adipocytes were treated with glucosamine (10 mM) for 24 h, and then palmitic acid oxidation was measured. Glucosamine treatment increased fatty acid oxidation in 3T3L1 adipocytes (160 ± 6%, p < 0.05, Fig. 3), which paralleled the activation of AMPKα and phosphorylation of ACC (Fig. 2). Conversely, blocking the hexosamine biosynthesis pathway with DON for 24 h significantly reduced fatty acid oxidation by 21% (p < 0.05) in 3T3L1 adipocytes. Glucosamine was able to bypass the inhibition of GFA by DON and stimulate fatty acid oxidation. These results in cultured 3T3L1 adipocytes are consistent with the transgenic mouse studies and demonstrate that activation of AMPK by chronic hexosamine flux stimulates fatty acid oxidation.
AMPK Is Required for Fatty Acid Oxidation Induced by the HBP in 3T3L1 Adipocytes—To confirm the role of AMPK in the HBP-mediated increase in fatty acid oxidation, we assessed the effect of adenovirus-mediated expression of a dominant negative form of AMPK in adipocytes. The catalytically inactive AMPKα2 (DN-AMPK) bearing a K45R mutation has been shown to function as a dominant negative inhibitor of both α1 and α2 AMPK activity (26, 33, 34). In control GFP-infected cells, the GFA inhibitor (DON) decreased phosphorylated AMPK, ACC, and palmitic acid oxidation (Fig. 4), similar to the results reported above from uninfected 3T3L1 adipocytes. However, infection of adipocytes with the DN-AMPK virus increased expression of the mutant AMPKα2 subunit and suppressed levels of phosphorylated AMPK and ACC (Fig. 4B). Similar to the effect of GFA inhibition, cells expressing the DN-AMPK displayed a 31% decrease in palmitic acid oxidation in the basal state (Fig. 4A). The GFA inhibitor led to no further inhibition of fatty acid oxidation. Glucosamine significantly enhanced fatty acid oxidation in control GFP-infected 3T3L1 adipocytes, and this was reduced by 32% ($p < 0.05$) in the cells expressing DN-AMPK. These data suggest that AMPK activation is required for chronic hexosamine flux to stimulate fatty acid oxidation in adipocytes.

Activation of AMPK by the HBP Is Not Associated with Changes in Cellular Adenine Nucleotides—AMPK is a sensor of AMP levels in cells, and its activity is dependent on the cellular AMP/ATP ratio (17, 19). To determine if changes in nucleotide concentration mediate the effects of the HBP on AMPK activation, we measured the levels of adenine nucleotides in fat pads from control and GFA transgenic mice (Table 1). Overexpression of GFA in fat pads did not alter the cellular levels of AMP, ADP, and ATP or the AMP/ATP ratio (0.161 ± 0.013 versus 0.170 ± 0.009, $p = 0.61$), demonstrating that AMPK activation in aP2-GFA mice is AMP-independent. The same is true in the cultured 3T3L1 adipocytes. The GFA inhibitor (DON) did not significantly affect the content of adenine nucleotides or the AMP/ATP ratio, although it reduced AMPK activity (Fig. 2B). Furthermore, glucosamine at concentrations of 0.5 mM (data not shown) and 1 mM (Table 1), which stimulate AMPK activation and ACC phosphorylation (Fig. 2A), also did not change the cellular AMP/ATP ratio. Higher concentrations of glucosamine (10 mM) did deplete cellular ATP by 44%, as has been reported (35–37).

O-Linked Glycosylation Is Involved in Regulation of AMPK Activation by the HBP—Recent data suggest that O-GlcNAc modification is the major mechanism mediating the cellular actions of the HBP, and in many cases, this modification directly alters protein activity (10, 38). To examine whether up-regulation of AMPK activity by the HBP is mediated through this O-GlcNAc modification, we used sWGA, a modified lectin that binds O-GlcNAc, to precipitate O-GlcNAc-modified proteins from 3T3L1 cell lysates. AMPKα was detected in sWGA precipitates (Fig. 5A). Furthermore, activation of the HBP by glucosamine increased AMPK protein levels in sWGA precipitates (156 ± 12%, $p < 0.01$). Conversely, blocking the HBP with the GFA inhibitor (DON) reduced sWGA-associated AMPK protein (55 ± 10%, $p < 0.02$). As predicted, however, the GFA inhibitor did not affect the glucosamine-induced increase in AMPK in lectin precipitates. Consistent with data from the cultured 3T3L1 adipocytes, AMPK was present in sWGA precipitates from wild type mouse fat pads, and this level was significantly higher in fat pads from GFA transgenic mice (146 ± 12%, $p < 0.05$) (Fig. 5B).

To determine if the changes in AMPK binding to sWGA were secondary to changes in AMPK phosphorylation, we
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TABLE 1
Adenine nucleotides in adipocytes

<table>
<thead>
<tr>
<th>Fat pads</th>
<th>3T3L1 adipocytes</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>AMP</td>
<td>0.310 ± 0.038</td>
</tr>
<tr>
<td>ADP</td>
<td>0.880 ± 0.085</td>
</tr>
<tr>
<td>ATP</td>
<td>1.840 ± 0.136</td>
</tr>
<tr>
<td>AMP/ATP Ratio</td>
<td>0.170 ± 0.009</td>
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*p < 0.05, ATP in GlcN (10 mM) versus control.

Our data suggest that O-linked glycosylation is involved in AMPK regulation. To test this hypothesis directly, sWGA precipitates from 3T3L1 adipocytes were treated in vitro with hexosaminidase to remove O-GlcNAc from protein. We found that removal of O-GlcNAc reduced AMPK activity in sWGA precipitates by 45% (Fig. 6A). Furthermore, hexosaminidase treatment also reduced immunoprecipitated AMPKα1 activity by 51% (Fig. 6B). Importantly, hexosaminidase treatment did not change the phosphorylation state of AMPK. These data strongly support the conclusion that O-linked glycosylation is directly involved in AMPK activation.

DISCUSSION

Our data demonstrate that chronic hexosamine flux stimulates fatty acid oxidation in adipocytes through activation of AMPK. We found higher AMPK activity, and consequently ACC phosphorylation, in transgenic fat pads overexpressing GFA, the rate-limiting enzyme of the HBP. Additionally, glucosamine treatment (24 h) of 3T3L1 adipocytes increased AMPK activity and fatty acid oxidation, and blocking hexosamine flux by inhibition of GFA reduced both AMPK activation and fatty acid oxidation. Dominant negative AMPK diminished the glucosamine-induced increase in ACC phosphorylation and fatty acid oxidation, which further confirms that this HBP-mediated fatty acid oxidation is mediated by AMPK activation. These data also indicate a novel mechanism wherein the HBP-induced O-linked glycosylation is involved in AMPK regulation.

AMPK is a metabolic master switch that senses change in energy status and plays a crucial role in maintaining cellular energy and metabolic homeostasis (17–19, 21). AMPK is activated by energy insufficiency following metabolic and nutritional stresses, including hypoxia and prolonged exercise, and responds by adjusting the rates of processes that consume or generate ATP. Since the HBP serves as a glucose sensor and activation of the HBP signals excess nutrient flux, it was expected that activation of the HBP might mirror energy sufficiency and therefore inhibit AMPK activity. Surprisingly, our current data demonstrate that chronic hexosamine flux increases AMPK activity, and consequently fatty acid oxidation, in both cultured 3T3L1 adipocytes and transgenic fat pads. This apparent paradox is probably explained by the differences between acute and chronic fuel excess. Our current studies were designed to mimic a situation of chronic fuel excess through constitutive overexpression of the rate-limiting enzyme (GFA) in hexosamine syn-

FIGURE 5. Chronic hexosamine flux increases AMPK protein in sWGA precipitates from adipocytes. A, differentiated 3T3L1 adipocytes were cultured in DMEM containing 1% fetal bovine serum in the presence or the absence of GFA inhibitor (DON; 10 μM) for 24 h. To chronically activate the HBP, cells were cultured in medium containing 2.5 mM glucose and 10 mM GlcN for 24 h. Cell lysates were precipitated with sWGA, followed by immunoblotting with anti-AMPKα. The lower panel shows the densitometric data from three independent experiments.* p < 0.01 GlcN treatment versus control treatment; #, p < 0.02 GFA inhibitor treatment versus control treatment. B, tissue homogenates from fat pads of wild type (WT) and transgenic mice (aP2-GFA) were precipitated with sWGA, followed by immunoblotting with anti-AMPKα. The lower panel shows densitometric data from three independent experiments. *, p < 0.01 aP2-GFA versus wild type. C, differentiated 3T3L1 adipocytes were treated with glucosamine (10 mM) for 24 h. Cell lysates were incubated with or without the recombinant catalytic subunit of protein phosphatase-1α (PP1α; 7 units) for 30 min at 37 °C. The lysates were precipitated with sWGA followed by immunoblotting for phosphorylated and total AMPKα.

treated 3T3L1 cell lysates with the catalytic subunit of PP1α to reduce the AMPK phosphorylation. PP1α reduced phosphorylation of AMPK as expected but did not affect the level of sWGA-bound AMPK (Fig. 5C). Thus, phosphorylation on AMPK does not modify its affinity for sWGA.
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Thesis or long term glucosamine treatment (24 h). There is evidence that effects of acute glucose exposure on AMPK differ from those of chronic glucose exposure. Raile et al. (39), for example, found inhibition of AMPK activity in INS-1E cells after short term high glucose treatment (10 min), as would be expected from an acute effect mediated by an increase in cellular energy status. However, the same investigators found that this inhibition was lost with prolonged treatment (24 h) with high glucose (39). We have also observed that short term glucose treatment (30 min) inhibits AMPK activity in 3T3L1 adipocytes (data not shown). As would be expected, this AMPK inhibition by acute glucose treatment is accompanied by a drop in the cellular AMP/ATP ratio (40, 41).

Adipose tissue functions primarily to store rather than utilize fuel, so it might exhibit unique regulatory mechanisms compared with other tissues in response to activation of the HBP or other stimuli. For example, activation of AMPK promotes glucose transport into skeletal muscle (42) but reduces insulin-stimulated glucose transport in adipocytes (30). The net result is to make fuel available to tissues requiring energy while limiting substrate availability for long term storage of energy. Similar tissue-specific effects on AMPK activity are seen with endocannabinoids and ghrelin, which increase AMPK activity in the hypothalamus and heart muscle while inhibiting AMPK in adipose tissue and liver (43). The HBP effects on energy metabolism are also tissue-specific; Rossetti’s group (4) demonstrated that activation of the HBP inhibited the expression of mitochondrial proteins involved in oxidative phosphorylation and substrate oxidation in skeletal muscle but increased the expression of these same oxidative phosphorylation genes in brown adipose tissue. Therefore, although chronic activation of the HBP activates AMPK and stimulates fatty acid oxidation in adipose tissue, the HBP may have distinct effects on AMPK in other tissues.

The best described pathway for AMPK activation involves an increase in the AMP/ATP ratio (18). We found several lines of evidence, however, demonstrating that the activation of AMPK by the HBP is not mediated by the AMP/ATP ratio. First, we observed that a chronic increase in hexosamine flux in transgenic fat pads stimulated AMPK activity without a change in cellular energy status. Second, inhibition of GFA activity reduced AMPK activity in 3T3L1 adipocytes without altering cellular levels of adenine nucleotides and AMP/ATP ratio. Third, glucosamine at concentrations of 0.5 and 1 mM also increased the HBP and AMPK activity in 3T3L1 adipocytes without an effect on the AMP/ATP ratio. The lack of an effect of low concentrations (up to 2 mM) of glucosamine on cellular ATP has been confirmed in other systems (35, 36). Consistent with published data (36, 37), glucosamine at the high concentration (10 mM) did increase the AMP/ATP ratio in 3T3L1 adipocytes, and this may contribute to further activation of AMPK that we observed with 10 mM glucosamine (Fig. 2). It is also worth noting that AMPK complexes containing the α1 isoform, the predominant isoform in adipose tissue, are less sensitive to AMP (44). Together, our data provide compelling evidence that chronic hexosamine flux in fat regulates AMPK activity by an AMP-independent mechanism. Consistent with this, recent evidence suggests that AMPK can be activated in response to stimuli that do not produce a measurable increase in intracellular AMP. For example, hyperosmotic stress (45), metformin (46), and long-chain fatty acids (47) activate AMPK without significant changes in the AMP/ATP ratio. Carling’s group (48) and Hardie’s group (49) also recently demonstrated that AMPK can be activated by calmodulin-dependent protein kinase kinase via a Ca\(^{2+}\)-dependent, AMP-independent mechanism.

We have previously shown that the levels of O-GlcNAc protein modification were increased in the aP2-GFA transgenic fat pads and glucosamine-treated 3T3L1 adipocytes (7, 14). The present study demonstrates that AMPK is found in O-GlcNAc-specific lectin precipitates, and its level is responsive to changes in hexosamine flux and AMPK activity. These results strongly suggest that O-linked glycosylation has a role in hexosamine-mediated AMPK regulation. The precise mechanism for
O-GlcNAc modulation of AMPK activation is not known, but it is likely to be multifactorial. Removal of O-GlcNAc in vitro by hexosaminidase significantly reduces AMPK activity (Fig. 6), suggesting that O-linked glycosylation, like Thr\(^{172}\) phosphorylation of AMPK, could increase AMPK activity directly. Since O-GlcNAc modification parallels the phosphorylation and activity of AMPK, it is also possible that O-GlcNAc modification could increase AMPK phosphorylation by making AMPK a better substrate for its upstream kinases, such as LKB1, or increasing the activity of its upstream kinase itself. The latter is somewhat less unlikely, because LKB1 is regarded as constitutively active (17, 50, 51), and LKB1 has not been demonstrated to be O-GlcNAc modified (data not shown). Regardless of the explanation, it is important to note that O-GlcNAc modification is involved in AMPK regulation. We were not directly able to detect O-GlcNAc modification of AMPK in immunoprecipitates (data not shown). However, the O-GlcNAc antibody used for detection (CTD110.6 antibody (52)) can be insensitive in detecting proteins without multiple-O-GlcNAc moieties. It is also possible that an AMPK binding partner, not AMPK itself, might be glycosylated.

The hexosamine pathway regulates metabolism through the mechanism of O-linked glycosylation of other metabolic enzymes. For example, Parker et al. (14) demonstrated that chronic activation of the hexosamine pathway induced O-GlcNAc modification of glycogen synthase and therefore inhibited its activity. Du et al. (53) and Federici et al. (54) have shown that eNOS is modified at Ser\(^{1177}\) by O-GlcNAc in hyperglycemic conditions. Ser\(^{1177}\) is the site that would otherwise be phosphorylated by AKT, leading to eNOS activation (55). Glycosylation, however, results in a reduction in eNOS activity. These observations, along with ours, support a paradigm of a nutrient sensing mechanism coupled to metabolic regulation wherein O-glycosylation acts as a damping influence on acute phosphate-mediated signaling.

In conclusion, we have demonstrated that chronic hexosamine flux stimulates AMPK activity, which leads to increased fatty acid oxidation in adipocytes. This adds to a growing body of evidence that the HBP is used by cells to sense nutrient status and coordinate metabolic changes, particularly in the face of chronic fuel sufficiency/excess. Our current discovery of a link between the HBP and AMPK signaling provides a model for future studies of how the HBP may interact with and influence other nutrient-dependent pathways.

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Hexosamine Biosynthesis Pathway Regulates AMPK Activity

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Chronic Hexosamine Flux Stimulates Fatty Acid Oxidation by Activating AMP-activated Protein Kinase in Adipocytes
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