Hexosamine biosynthesis pathway flux promotes endoplasmic reticulum stress, lipid accumulation, and inflammatory gene expression in hepatic cells

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Sage AT, Walter LA, Shi Y, Khan MI, Kaneto H, Capretta A, Werstuck GH. Hexosamine biosynthesis pathway flux promotes endoplasmic reticulum stress, lipid accumulation, and inflammatory gene expression in hepatic cells. Am J Physiol Endocrinol Metab 298: E499–E511, 2010. First published December 1, 2009; doi:10.1152/ajpendo.00507.2009. —There is increasing evidence that endoplasmic reticulum (ER) stress contributes to the development of atherosclerosis in diabetes mellitus. The purpose of this study was to determine the effects of increased hexosamine biosynthesis pathway (HBP) flux on ER stress levels and the complications of ER stress associated with diabetes and atherosclerosis in hepatic cells. Glutamine:fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme of the HBP, was overexpressed in HepG2 cells by use of an adenoviral expression system. The ER stress response and downstream effects, including activation of lipid and inflammatory pathways, were determined using real-time PCR, immunoblot analysis, and cell staining techniques. GFAT overexpression resulted in increased expression of ER stress markers, including Grp78, Grp94, calreticulin, and GADD153, relative to cells infected with an empty adenoviral vector. In addition, GFAT overexpression promoted lipid, but not cholesterol, accumulation in hepatic cells as well as inflammatory pathway activation. Treatment with 6-diazo-5-oxo-<wbr/>norleucine, a GFAT antagonist, blocked the effects of GFAT overexpression. Consistent with our in vitro data, hyperglycemic mice presented with elevated markers of hepatic ER stress, glucosamine and lipid accumulation. Together, these data suggest that HBP flux-induced ER stress plays a role in the development of hepatic steatosis and atherosclerosis under conditions of hyperglycemia.

Diabetes mellitus; atherosclerosis; glucosamine; glutamine: fructose-6-phosphate amidotransferase

The incidence of diabetes mellitus (DM) is increasing worldwide. DM is associated with a number of debilitating complications including amputation, retinopathy, nephropathy, peripheral neural damage, and cardiovascular disease. Cardiovascular disease accounts for over 70% of all diabetic mortalities (11). However, the molecular mechanisms linking diabetes and hyperglycemia to micro- and macrovascular diseases including atherosclerosis are not completely understood.

Recently, our laboratory (49) and others’ (52) have presented evidence for a novel mechanism by which hyperglycemia-induced endoplasmic reticulum (ER) stress promotes the activation of proatherogenic pathways. In eukaryotic cells, the ER provides a contained environment for the synthesis and modification of membrane-associated and secreted proteins. Co- and posttranslational modifications, including disulfide bond formation and protein glycosylation, play an important role in the folding and processing of proteins (15). ER stress arises when agents or conditions that disrupt ER homeostasis cause the accumulation of unfolded or misfolded proteins (reviewed in Refs. 36, 40, 42).

In mammalian cells, conditions of ER stress lead to the initiation of the unfolded protein response (UPR), which functions to return cells to homeostasis by 1) the general inhibition of protein translation and 2) the specific induction of ER-resident chaperone expression, including Grp78, Grp94, and calreticulin (CALR) (7, 13, 24). These ER chaperones mediate protein folding through interactions that stabilize nascent proteins. Failure of the ER to return to homeostasis during conditions of prolonged ER stress can result in the activation of proapoptotic pathways that are mediated by the effector protein GADD153/CHOP (growth arrest DNA damage-inducible/C/EBP homologous protein) (54).

There is increasing experimental evidence in support of a direct and causative role for ER stress in the development and/or progression of diabetes mellitus, particularly with respect to pancreatic β-cell death (2, 19, 34, 44) and insulin resistance (35). In recent years, ER stress has been linked to the development of complications associated with diabetes mellitus, including nephropathy, retinopathy, and cardiovascular complications (18, 23, 29, 49).

Results from our laboratory have implicated intracellular glucosamine accumulation in hyperglycemia-induced atherosclerosis (4, 49, 50). Under physiological conditions, 1–3% of intracellular glucose is shunted from the glycolytic pathway to the hexosamine biosynthesis pathway (HBP), and flux through the HBP increases with glucose concentration (5, 10, 17, 45). Glutamine:fructose-6 phosphate amidotransferase (GFAT) is the rate-limiting enzyme of the HBP responsible for the conversion of l-glutamine and d-fructose-6-phosphate to l-glutamate and d-glucosamine 6-phosphate (GlcN-6-P) (reviewed in Ref 31). GlcN-6-P is subsequently converted into UDP-N-acetyl glucosamine (UDP-GlcNAc), which acts as a substrate for N- and O-linked protein glycosylation (9). Glycosylation is essential to the folding, translocation, function, and stability of many proteins, and GFAT plays a central role in facilitating these processes. Dysregulation of GFAT has been associated with diabetic complications including insulin resistance in muscle and adipose tissues and renal dysfunction (5). Although evidence exists that improper glycosylation via the HBP disrupts key cellular processes, the potential role of the HBP in particular GFAT, in hyperglycemia-induced ER stress has not been examined.

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In this study, we investigated the effect(s) of GFAT overexpression on 1) HBP flux, 2) the induction of ER stress, and 3) activation of proatherogenic pathways, including lipid biosynthesis and inflammatory gene expression in hepatic cells. Finally, we investigate the relationship between the HBP and ER stress in hyperglycemic apoE−/− mice.

MATERIALS AND METHODS

Virus purification. Ad-GFAT-GFP contains a CMV promoter driving the expression of a polycistronic message containing the GFP coding region, an internal ribosome entry site (IRES), followed by the GFAT coding region. Ad-GFP is identical but lacks the GFAT cDNA. Viruses were amplified in HeLa293 cells (ATCC, Manassas, VA) and purified using the Adeno-X Virus Mini Purification Kit (Clontech, Mountain View, CA). Viral titers were determined using a standard plaque assay in HeLa293 cells.

Cell culture and treatment. Human hepatocarcinoma cells (HepG2) (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Burlington, ON, Canada) containing 10% FBS and 5 mM glucose. Cells were infected 24 h following plating with Ad-GFP or Ad-GFAT-GFP at an MOI of 100. After 60 min, the cells were rinsed in DMEM and cultured for an additional 48 h at 37°C with 5% CO2. Infection efficiencies were determined by visualizing GFP expression using a Zeiss AxioScope 2 microscope. Images were captured with a Canon Power Shot S315. Following infection, infected cells were pretreated with 20 μM DON (Sigma-Aldrich, Oakville, ON, Canada) for 60 min. All cells were then cultured in 30 mM glucose for 8–72 h at 37°C with 5% CO2.

Immunoblot analysis. Equal amounts of total protein lysates were resolved by SDS-PAGE under reducing conditions. Proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) and immunoblotted with mouse polyclonal GFAT (1:3,000; provided by Dr. Cora Weigert, University of Tubingen), GFP (1:20,000; Clontech), RL2 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), KDEL (Lys-Asp-Glu-Leu, 1:1,000; Stressgen), HMG-CoA reductase (1:1,000, Millipore, Billerica, MA), or PARP (poly-ADP-ribose polymerase, 1:1,000; Cell Signaling, Danvers, MA). A horseradish peroxidase-conjugated goat anti-rabbit IgG or sheep anti-mouse IgG (Dako, Mississauga, ON, Canada) were used as secondary antibodies. Bands were visualized using enhanced chemiluminescence (Millipore).

GFAT activity assay. GFAT activity was determined using a procedure adapted from Badet et al. (28). The assay was performed in a 96-well plate using 20 μl of incubation mixture [100 mM L-glutamine (Sigma Aldrich), 100 mM fructose-6-phosphate (Sigma Aldrich), 50 mM EDTA (EMD Chemicals, Gibbstown, NJ), 10 mM DTT]. Recombinant GFAT (kindly provided by Dr. Bernard Badet, CNRS) was used as a positive control. The plate was sealed and images were visualized using a Leica DMIL inverted microscope and captured with a Canon Power Shot S315 and quantified using ImageJ software.

Hyperglycemic mouse model. The McMaster University Animal Research Ethics Board approved all procedures. Five-week-old apoE-deficient (B6.129P2-ApoEtm1Unc) mice were fed a standard chow diet (TD92078, Harlan Teklad) and then randomly divided into two groups. Mice were injected intraperitoneally over 5 consecutive days with either 40 mg/kg streptozotocin (STZ, Sigma) or an equal volume of citrate buffer as control. Injections were repeated at 7 wk of age. Mice were killed at 15 wk of age, and liver was flash-frozen or perfusion-fixed with 10% neutral buffered formalin. Frozen sections were stained with or Oil Red O as described above. The Vectastain ABC system (Vector Laboratories, Burlingame, CA) was used to stain sections with the appropriate primary and biotinylated secondary antibodies. Non-specific staining was controlled for by using a similar section and preimmune IgG. Images were captured with a charged-coupled device color video camera (Sony).

Statistical analysis. All data are presented as means ± SE. Significant differences in the sample groups were established using the unpaired Student’s t-test, with P < 0.05 being considered statistically significant.

RESULTS

Adenovirus-mediated GFAT overexpression in HepG2 cells. HepG2 cells were infected with Ad-GFAT-GFP at an MOI of 100 for 48 h. To monitor for the possible effects of adenoviral infection, HepG2 cells were infected, under identical conditions, with a control vector that contained GFP but not GFAT cDNA (Ad-GFP). Productive infection was monitored by visualizing GFP expression by fluorescence microscopy (Fig. 1, A and B). Total RNA was isolated from infected cells, and GFAT mRNA levels were determined by real-time PCR analysis. GFAT transcript levels were more than 10-fold higher (P < 0.05) in Ad-GFAT-GFP infected cells than in controls (Fig. 1C). Immunoblotting
Analysis was performed on total protein lysates prepared 48 h postinfection (Fig. 1D). Both Ad-GFP- and Ad-GFAT-GFP-infected cells showed robust GFP expression (Fig. 1E). Ad-GFAT-GFP- but not Ad-GFP-infected cells showed a significant, 3.0-fold increase ($P < 0.05$) in GFAT protein levels (Fig. 1F). GFAT overexpression leads to increased activity of the HBP. Next, we investigated whether GFAT overexpression at the mRNA and protein levels corresponded with an increase in GFAT activity and, in particular, increased HBP activity. The intracellular concentration of GlcN-6-P was estimated by mon-
itoring the extent of O-GlcNAc modification of the nuclear pore protein p62, as previously described (49). Protein lysates from Ad-GFAT-GFP-infected cells showed a significant increase (2.4-fold, \( P < 0.05 \)) in the levels of O-linked glycosylation compared with the controls (Fig. 2, A and B). We also directly measured GFAT activity using the Elson-Morgan method to monitor GlcN-6-P production. In Ad-GFAT-GFP-infected cells, GFAT activity was increased 1.9-fold (\( P < 0.001 \)) compared with control cells (Fig. 2C). To verify that the signal measured by the Elson-Morgan assay was, in fact, a result of GFAT activity, we used a selective inhibitor of GFAT, DON. Protein lysates from Ad-GFAT-GFP-infected HepG2 cells pretreated for 1 h with 20 \( \mu \)M DON showed a significant reduction in activity (\( P < 0.01 \); Fig. 2C).

**GFAT overexpression and glucosamine accumulation are associated with UPR activation.** To investigate the effects of GFAT overexpression on ER stress and the UPR, we measured specific mRNA transcript levels using real-time PCR. Total RNA was isolated 8, 24, and 48 h after infection with Ad-GFP or Ad-GFAT-GFP and treatment with 30 mM glucose. Primers specific for ER stress response genes, including Grp78, Grp94, CALR, GADD153/CHOP, and the control gene \( \beta \)-actin were used to amplify and quantify specific gene products. After 8 h, there was a 9.8-fold increase in Grp78, a 4.9-fold increase in Grp94, and a 4.4-fold increase in CALR (\( P < 0.05 \); Fig. 3A). After 24 h, Grp78 was induced 5.6-fold over controls, and there was no significant induction after 48 h (Fig. 3, B and C, \( P < 0.05 \)). Grp94 showed no significant induction at either 24 or 48 h, whereas CALR was elevated at both time points (3.3- and 4.6-fold respectively; Fig. 3, B and C, \( P < 0.05 \)). GADD153/CHOP was induced only after 48 h (11.6-fold, \( P < 0.05 \)), which is consistent with prolonged ER stress and the activation of proapoptotic pathways (Fig. 3C). Pretreatment with DON significantly attenuated the induction of Grp78 and CALR (\( P < 0.05 \); Fig. 3, D and E, respectively).

We next examined the effect of GFAT overexpression on the induction of specific proteins that are diagnostic markers of the UPR. Immunoblot analysis was used to determine the protein levels of Grp78, Grp94, and CALR in Ad-GFAT-GFP- or Ad-GFP-infected HepG2 cells (Fig. 4, A–C). Pretreatment with DON significantly attenuated the induction of Grp78 and Grp94 (\( P < 0.05 \)) in cells treated with Ad-GFAT-GFP.

Finally, to correlate the effects of GFAT overexpression to the previously observed effects of exogenous glucosamine, we treated HepG2 cells with increasing glucosamine concentrations (0 to 30 mM) for 8 h. HepG2 cells treated with \( \geq 0.6 \) mM glucosamine showed a significant induction of Grp78 (\( P < 0.05 \); Fig. 4D) and Grp94 protein levels (\( P < 0.05 \); Fig. 4E). Adenovirus-mediated GFAT overexpression correlated with exposure to 2.5–5 mM exogenously added glucosamine with respect to the induction of Grp78 and Grp94 protein levels. To control for possible nonspecific effects of DON on the ER stress response, we pretreated cells with DON before treatment with 1 mM glucosamine for 8 h. There were no significant changes on Grp78 protein levels in cells pretreated with DON (\( P = 0.9 \); Fig. 4F). **GFAT overexpression is associated with PARP activation.** The ER stress response gene GADD153 is associated with the activation of proapoptotic pathways. We investigated the possible correlation of GFAT overexpression and the proteolytic cleavage/activation of the proapoptotic protein PARP by using immunoblotting techniques. After a 72-h treatment with 30 mM glucose, HepG2 cells overexpressing GFAT displayed significant elevations in the levels of cleaved PARP (1.8-fold, \( P < 0.05 \); Fig. 5). Pretreatment with the GFAT inhibitor DON effectively blocked PARP activation in GFAT overexpressing cells (\( P < 0.05 \); Fig. 5).
Overexpression of GFAT alters lipid metabolism. To investigate the downstream effects of GFAT overexpression, we analyzed several markers of lipid metabolism. After infection with Ad-GFAT-GFP for 48 h, HepG2 cells were treated with 30 mM glucose for 8 h. Total RNA was extracted, and real-time PCR analysis was performed for SREBP-1/2, FAS, and HMG-CoA reductase (Fig. 6, A–D). A significant induction of transcripts encoding SREBP-1 (6.7-fold, \( P < 0.05 \)) and SREBP-2 (2.1-fold, \( P < 0.05 \)) was observed. Pretreatment with DON was successful in ameliorating the induction of both SREBP-1 and -2 (\( P < 0.05 \)).

In accord with a large induction of SREBP-1, we observed an 18.6-fold induction of FAS above respective controls (\( P < 0.05 \)), and this response was attenuated by pretreatment with DON. There was only a marginal increase in HMG-CoA reductase mRNA, correlating with a relatively small induction of SREBP-2. However, at the protein level, the induction of HMG-CoA reductase was significant at 1.8-fold (Fig. 6 E).

**Overexpression of GFAT promotes lipid, but not free cholesterol accumulation.** After observing that there was a significant induction of SREBP-1 and related proteins (FAS), we wanted to investigate the effects of GFAT overexpression on intracellular triglyceride levels. HepG2 cells were infected under identical conditions, stimulated with 30 mM glucose for 48 h, and stained using Oil Red O. A subset of cells were pretreated with 20 \( \mu \)M DON or 4 mM valproate to inhibit GFAT activity or ER stress signaling, respectively. Images were captured using a Leica in-
verted microscope (Fig. 7A), and Oil Red O was extracted and read at 510 nm using a spectrophotometer. In cells overexpressing GFAT, we observed a 1.2-fold increase in intracellular triglyceride levels above controls after 48 h ($P < 0.05$; Fig. 7B). The accumulation of neutral lipids was attenuated by pretreatment with either DON or valproate ($P < 0.05$).

Additionally, we looked at whether the induction of SREBP-2 and HMG-CoA reductase had any effect on total unesterified cholesterol levels. We infected HepG2 cells for 48 h and treated them with 30 mM glucose for 24 and 48 h before staining for free cholesterol with filipin (Fig. 8A). Images were captured using the Leica inverted microscope and quantified for pixel intensity per area (Fig. 8, B and C). After both 24 and 48 h, there was no significant increase in unesterified cholesterol levels in cells expressing GFAT above controls.

Overexpression of GFAT induces inflammatory gene expression. We investigated the possible effect of GFAT overexpression and ER stress on the activation of proinflammatory gene expression. Real-time PCR was used to determine transcript levels of IL-1α/β, IL-6, and IL-8 after 24, and 48 h (Fig. 9, A and B). After 24 h, we observed a significant, 9.7-fold induction in IL-1α mRNA and a 19.5-fold increase in IL-8...
(P < 0.05). After 48 h, a 2.5-fold increase in IL-8 mRNA was observed in cells overexpressing GFAT (P < 0.05).

Hyperglycemic apoE-deficient mice exhibit hepatic ER stress and hepatic steatosis. Liver tissue from normoglycemic and STZ-induced hyperglycemic apoE-deficient mice was examined to investigate correlations between O-glycosylation, ER stress, and lipid accumulation. Chronically elevated blood glucose levels (20.2 ± 1.1 mM) in STZ-injected mice corresponded to increased hepatic protein O-glycosylation and activation of the ER stress response, as indicated by elevated KDEL staining (Fig. 10). Furthermore, livers from the diabetic apoE-deficient mice showed significant lipid accumulation as determined by Oil Red O staining (Fig. 10). This result is consistent with the observed HBP flux-induced ER stress and lipid accumulation in vitro.

DISCUSSION
We have previously shown that glucosamine, a product of the HBP, is a potent ER stress-inducing agent in cultured cells (25). HBP flux has been implicated in complications associated with diabetes mellitus, including insulin resistance and nephropathy (5); however, the potential role of the HBP, and GFAT specifically, as a modulator of hyperglycemia-induced ER stress has not been addressed. The present study demonstrates that adenovirus-mediated overexpression of GFAT in HepG2 cells can promote ER stress and the downstream cellular dysfunctions that have been associated with atherogenesis. Increased GFAT expression is correlated with significant elevation in levels of O-linked protein glycosylation. This result is consistent with increased HBP flux and subsequent glucosamine 6-phosphate biosynthesis. Cells overexpressing GFAT show significantly increased levels of diagnostic markers for ER stress at both the mRNA and protein levels that were attenuated by the GFAT inhibitor DON. GFAT overexpression in hepatic cells was associated with the dysregulation of lipid metabolism pathways. There was a significant induction of SREBP-1 and -2, which regulate the expression of enzymes involved in lipid biosynthesis and uptake. This translated to a significant increase in intracellular triglycerides, but no change in unesterified cholesterol levels. Finally, there was a significant increase in the transcript levels of several markers of inflammation in HepG2 cells overexpressing...
previously shown that culturing cells in 1–5 mM glucosamine linked glycosylation at the ER membrane. We (49) have demonstrated that GlcNAc, promotes protein misfolding by interfering with the stability of proteins including apoB-100 and α1-antitrypsin. Further research is required to determine the mechanism by which HBP activity and UDP-GlcNAc accumulation may alter LLO production and/or protein N-glycosylation.

The liver plays a central role in the metabolism and regulation of plasma glucose and lipids. Specifically, hepatocytes act as major centers for gluconeogenesis, cholesterol and triglyceride synthesis, and glucose and lipoprotein particle secretion into the circulation as well as a removal of lipids in the form of low-density lipoprotein particles from the blood. Together these functions are essential to the overall maintenance of energy homeostasis. Patients with diabetes mellitus commonly present with dyslipidemia consisting of hypertriglyceridemia and a reduction in high-density lipoprotein levels (6, 30, 46). In addition, hepatic steatosis is often found in individuals with type 2 diabetes (26). It has been previously demonstrated that homocysteine-, tunicamycin-, and thapsigargin-induced ER stress can activate SREBPs and promote lipid accumulation (27, 51). Our data suggest that HBP flux, activated in the presence of hyperglycemia, can promote ER stress and subsequently lead to the activation of SREBPs. Hence, HBP flux and ER stress may represent an underlying cause of hepatic steatosis in patients with diabetes mellitus and, through disruption of lipid transport systems, may promote the progression of atherosclerosis.

In our study, GFAT overexpression promoted HBP flux and a robust induction of SREBP-1 and FAS mRNA expression, which led to a significant increase in intracellular neutral lipids after 48 h. Using an inhibitor of GFAT, DON, and an inhibitor of downstream ER stress signaling, valproate (4), we were able to effectively attenuate the accumulation of neutral lipids, supporting a causative role for ER stress in this effect. Rumberger et al. (41) previously demonstrated that the HBP can drive lipogenesis under hyperglycemic conditions. Our data not only support these findings but identify the activation of ER stress response pathways as the mechanism by which hyperglycemia may promote lipid accumulation. Taken together, the results presented here, along with previously reported findings from our laboratory and others7, suggest that the HBP and ER stress may play a role in the dysregulation of hepatic function (23, 49, 50).

We (25) previously showed that HepG2 cells cultured in the presence of 5 mM glucosamine accumulate unesterified cholesterol as well as neutral lipids. In this study, we did not detect changes in intracellular unesterified cholesterol levels. This observation is consistent with the modest increases in cholesterol biosynthetic enzyme expression that we detected under conditions of increased HBP flux. It is possible that, in contrast to conditions of exogenous glucosamine-induced ER stress, the cholesterol esterification capacity of HepG2 cells is not overwhelmed under conditions of HBP-associated ER stress. Thus, any accumulation of unesterified cholesterol associated with GFAT overexpression can be rapidly esterified by acyl-CoA cholesterol acyltransferase (ACAT).

ER stress-inducing agents have been shown to activate the expression of proinflammatory factors, and IL-8 has specifically

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Fig. 5. GFAT overexpression promotes poly-ADP-ribose polymerase (PARP) cleavage in HepG2 cells. HepG2 cells were infected with Ad-GFP or Ad-GFAT-GFP (MOI 100) for 48 h and then cultured in 30 mM glucose in the presence or absence of 20 μM DON for 72 h. Total protein lysates were resolved by SDS-PAGE and immunoblotted with an antibody against PARP. Protein levels were quantified and normalized to β-actin loading control (**P < 0.05 relative to untreated and Ad-GFP controls, n = 3–5, ***P < 0.05 relative to the same treatment in the presence of 20 μM DON, n = 3).

GFAT. These in vitro data are supported by our observations from an STZ-injected apoE−/− mouse model. We found that hyperglycemic mice exhibit increased hepatic protein O-glycosylation, elevated levels of ER stress, and significant hepatic lipid accumulation relative normoglycemic controls. Taken together, flux through the HBP promotes ER stress and causes cellular dysfunctions that underlie physiological disorders that are associated with diabetes mellitus, including hepatic steatosis and atherosclerosis.

Previous studies have shown that overexpression of GFAT can promote insulin resistance and alter gene expression in a manner that is independent of increased glucose concentration (5, 14, 21, 48). In our experiments, GFAT overexpression induced a significant increase in the ER stress response only in the presence of elevated concentrations of glucose. This observation may be an indication that there is a direct correlation between the magnitude of the flux through the HBP and the severity of the downstream effect. More experiments will be required to further investigate this possibility.

The precise mechanism by which increased HBP flux promotes ER stress has yet to be worked out in detail. However, we propose that the accumulation of the HBP product, UDP-GlcNAc, promotes protein misfolding by interfering with N-linked glycosylation at the ER membrane. We (49) have previously shown that culturing cells in 1–5 mM glucosamine potently induces ER stress in HepG2 and other cell types.

Tunicamycin, a potent ER stress inducer, acts by inhibiting lipid-linked oligosaccharide (LLO) synthesis (43). Furthermore, Qiu et al. (37) have shown that elevated concentrations of glucosamine disrupt the N-linked glycosylation, processing, and stability of proteins including apoB-100 and α1-antitrypsin. Further research is required to determine the mechanism by which HBP activity and UDP-GlcNAc accumulation may alter LLO production and/or protein N-glycosylation.
been shown to respond to conditions of ER stress (16). The induction of IL-8 and IL-1β in HepG2 cells overexpressing GFAT after 24 and 48 h supports the role of the HBP and ER stress in mediating inflammation. Our results suggest that the induction of inflammatory factors is selective because no significant change was observed in the levels of IL-6 or IL-1β.

Furthermore, inflammatory pathway activation and lipid accumulation are hallmark characteristics of atherosclerosis, and here we suggest that, in patients with diabetes mellitus, they are induced as a result of HBP flux and ER stress.

There is accumulating experimental evidence in support of a direct and causal role for ER stress and the activation of the UPR in the development of diabetes and diabetes-associated complications. Secretory cells, including insulin-producing pancreatic β-cells, are dependent on efficient ER function to facilitate protein processing and export. In the Akita mouse model of diabetes, aberrations in proinsulin folding resulting from a point mutation in the Ins2 gene (C96Y), can disrupt disulfide bond formation in insulin processing (47). This results in the accumulation of misfolded proteins in the ER and ultimately β-cell death (1, 33). Although these findings suggest a potential role for ER stress as a causative agent in diabetes associated complications, the physiological relevance of these observations is demonstrated in the provided experiments.

Fig. 6. Effect of GFAT overexpression on lipid metabolism in HepG2 cells. HepG2 cells were infected for 48 h with Ad-GFP or Ad-GFAT-GFP (MOI 100). A subset of cells was pretreated with 20 μM DON. Total RNA was isolated, and real-time PCR was used to quantify mRNA levels of SREBP-1 (A) and SREBP-2 (B), fatty acid synthase (FAS) (C), and HMG-CoA reductase (D), as indicated (*P < 0.05 relative to controls, **P < 0.05 relative to the same treatment in the absence of DON). E: total protein lysates from identically treated cells were resolved by SDS-PAGE and immunoblotted with an antibody against HMG-CoA reductase. Protein levels were quantified and normalized to β-actin loading control (*P < 0.05 relative to controls, n = 3).
unclear. Our results are the first to demonstrate that increased HBP flux can induce the ER stress response and the UPR. Furthermore, we present evidence that supports the relevance of this pathway in vivo, in the development of hepatic steatosis.

Because GFAT is expressed in all cells of the body (8, 32), the effects of increased HBP flux may be relevant to other cell types and may be responsible for hyperglycemia-associated cellular dysfunctions in other tissues. In fact, previous reports have associated GFAT overexpression to activation of proatherosclerotic pathways in mesangial and vascular smooth muscle cells (22, 39). The UPR is a complex, multifaceted, and often cell-specific response that consists of a balance of protective and proapoptotic forces. Although we do not yet understand precisely how increased HBP flux disrupts ER homeostasis, there is evidence that glucosamine accumulation impairs N-linked glycosylation, an essential step in protein folding, in vitro (38).

In conclusion, this study supports a role for increased HBP flux in the development and progression of hyperglycemia-associated complications, including hepatic steatosis and atherosclerosis. Further studies will be required to determine the precise molecular mechanisms and identify additional tissuespecific effects that arise in conditions of diabetes mellitus and hyperglycemia.

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DISCLOSURES

No conflicts of interest are reported by the author(s).

REFERENCES


Fig. 7. GFAT overexpression promotes lipid accumulation in HepG2 cells. HepG2 cells were infected for 48 h with Ad-GFP or Ad-GFAT-GFP (MOI 100). A: cells were pretreated with 20 μM DON or 4 mM valproate and then supplemented with 30 mM glucose for 48 h and then stained with Oil Red O. B: intracellular Oil Red O was extracted and quantified by spectrometry (510 nm) [*P < 0.05 relative to controls, ** or #P < 0.05 relative to the same sample in the absence of 20 μM DON (**)) or 4 mM valproate (#), n = 3–4.


IL-1, IL-6, and IL-8 relative to HepG2 levels of IL-1 and real-time PCR used to quantify mRNA expression levels of IL-1α, IL-1β, IL-6, and IL-8 relative to β-actin mRNA levels, as indicated (*P < 0.05 relative to controls, n = 3).

Fig. 9. GFAT overexpression promotes increased levels of inflammatory factors in HepG2 cells. HepG2 cells were infected with Ad-GFP or Ad-GFAT-GFP (MOI 100) for 24 (A) and 48 h (B). Total RNA was isolated and reverse transcribed to detect GFAT mRNA overexpression. The levels of IL-1α, IL-1β, IL-6, and IL-8 were quantified by real-time PCR.

References:
37. Qin W, Avramoglou RK, Rutledge AC, Tsai J, Adeli K. Mechanisms of glucosamine-induced suppression of the hepatic assembly and secretion of...


