Excessive Hexosamines Block the Neuroprotective Effect of Insulin and Induce Apoptosis in Retinal Neurons

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In addition to microvascular abnormalities, neuronal apoptosis occurs early in diabetic retinopathy, but the mechanism is unknown. Insulin may act as a neurotrophic factor in the retina via the phosphoinositide 3-kinase/Akt pathway. Excessive glucose flux through the hexosamine biosynthetic pathway (HBP) is implicated in the development of insulin resistance in peripheral tissues and diabetic complications such as nephropathy. We tested whether increased glucose flux through the HBP perturbs insulin action and induces apoptosis in retinal neuronal cells. Exposure of R28 cells, a model of retinal neurons, to 20 mM glucose for 24 h attenuated the ability of 10 nM insulin to rescue them from serum deprivation-induced apoptosis and to phosphorylate Akt compared with 5 mM glucose. Glucosamine not only impaired the neuroprotective effect of insulin but also induced apoptosis in R28 cells in a dose-dependent fashion. UDP-N-acetylhexosamines (UDP-HexNAc), end products of the HBP, were increased ~2- and 15-fold after a 24-h incubation in 20 mM glucose and 1.5 mM glucosamine, respectively. Azaserine, a glutaminase:fructose-6-phosphate amidotransferase inhibitor, reversed the effect of 20 mM glucose, but not that of 1.5 mM glucosamine, on attenuation of the ability of insulin to promote cell survival and phosphorylate Akt as well as accumulation of UDP-HexNAc. Glucosamine also impaired insulin receptor processing in a dose-dependent manner but did not decrease ATP content. By contrast, in L6 muscle cells, glucosamine impaired insulin receptor processing but did not induce apoptosis. These results suggest that the excessive glucose flux through the HBP may direct retinal neurons to undergo apoptosis in a bimodal fashion; i.e. via perturbation of the neuroprotective effect of insulin mediated by Akt and via induction of apoptosis possibly by altered glycosylation of proteins. The HBP may be involved in retinal neurodegeneration in diabetes.

Diabetic retinopathy (DR) is usually considered a disease of the microvasculature, but significant involvement of neuronal components has been implicated as well. Previous studies by us and others (1, 2) indicate that neuronal cells in the retina, including ganglion cells, undergo apoptosis both in rats and humans with early diabetes. The pro-apoptotic BAX protein was also reported to be induced in neuronal as well as vascular components of the retina in patients with diabetes (3). However, the mechanism of the neurodegeneration in DR remains open to debate. Because insulin administration reduced the rate of apoptosis in streptozotocin-diabetic rats (2), systemic metabolic compromise such as hyperglycemia or defective insulin action, or both, adversely affects neuronal survival in the retina.

Insulin is known to act as a neurotrophic factor in cultured neuronal cells including retinal ganglion cells (4, 5). Insulin exerts a broad array of biological responses by binding to its specific receptors and activating the intracellular signaling cascades such as the IRS-1/PI3K/Akt pathway. Our recent findings have indicated that physiological concentrations of insulin rescue R28 cells, a model of retinal neurons (6–8), from apoptosis induced by serum withdrawal by activating the PI3K/Akt pathway, while inactivating caspase-3 (9). The neuronal components in the retina express abundant IR (10, 11). These observations suggest that insulin may play a critical role in maintaining neuronal survival in the retina.

Increased glucose flux through the HBP is thought to play a role in glucose-induced insulin desensitization in peripheral tissues and the development of diabetic complications such as nephropathy (12–26). The first and rate-limiting enzyme in this pathway, GFAT, catalyzes the conversion of fructose 6-phosphate to glucosamine 6-phosphate. The latter is rapidly metabolized to UDP-HexNAc, i.e. UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine, in an ~3:1 ratio. Although only up to 3% of glucose taken up by cells enters into the HBP, the end products UDP-HexNAc serve as essential substrates for the synthesis of glycosyl side chains of proteins and lipids (12–14). Thus, even modest perturbations of the amount of glucose flux through the HBP can exert diverse effects on protein functions. Glucosamine enters this pathway distal to GFAT (27) and induces insulin resistance in muscles and adipocytes (15–20). Increased ambient glucose concentration or

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‡ The abbreviations used are: DR, diabetic retinopathy; HBP, hexosamine biosynthetic pathway; IR, insulin receptor; IRS, insulin receptor substrate; IGF-I, insulin-like growth factor-1; PI3K, phosphoinositide-3-kinase; GFAT, glutamine:fructose-6-phosphate amidotransferase; DME, Dulbecco’s modified Eagle’s medium; UDP-HexNAc, UDP-N-acetylhexosamines; MOPS, 4-morpholino propane sulfonic acid; PAGE, polyacrylamide gel electrophoresis.
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exposure to glucosamine impairs insulin stimulation of Akt activity in fat cells (18), muscle (22, 23), liver (24), and rat-1 fibroblasts (28). On the other hand, the HBP has also been linked to glucose-mediated changes in cellular growth and growth factor expression. For example, high glucose stimulates transforming growth factor-β mRNA levels and extracellular matrix synthesis via the HBP in mesangial cells (25, 26), which is presumably associated with the development of diabetic nephropathy.

Under physiological conditions, endothelial and glial cells elegantly regulate glucose supply to the neuronal cells in the retina (29). However, DR adversely affects both glial and endothelial functions even at the early stages of DR (30–33). Intracellular concentrations of glucose are elevated in diabetic retinal tissues (34, 35). Thus, glucose metabolism in retinal neurons is likely to be perturbed under diabetic conditions. The retina expresses active GFAT (36) and synthesizes UDP-HexNAc (37). Hexosamine content is increased in retinal tissues in humans and rats with diabetes (38) and in the vitreous in alloxan-induced diabetic rabbits (39).

From the evidence presented above, we hypothesized that excessive glucose flux through the HBP and accumulation of UDP-HexNAc could reduce the neuroprotective effect of insulin or directly affect survival mechanisms in retinal neurons. To test this hypothesis, we investigated the effects of high glucose and glucosamine on insulin-mediated anti-apoptosis and IR processing and signaling in R28 cells. Our results indicate that high glucose and glucosamine prevent insulin from protecting R28 cells from apoptosis, which is associated with reduced insulin signaling and Akt activation. Furthermore, at higher concentrations glucosamine induces apoptosis even in serum-fed R28 cells.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents**—Azaserine, bovine insulin, glucosamine, and mannitol were purchased from Sigma. All other dry chemicals were purchased from Fisher unless otherwise stated. Transferrin-label metabolic reagents were from ICN Biomedicals (specific activity >1000 Ci/ml; 10 mCi/ml). DMEM with 1000 mg/liter glucose and metabolic labeling reagents were from ICN Biomedicals (specific activity 150 Ci/mmol); [3H]thyminine:1,1,2-trichlorofluoroethane (Sigma). The aqueous phase was stored at −80 °C and analyzed within 5 days. Nucleotide-linked hexoses and hexosamines were separated and measured by anion exchange high pressure liquid chromatography. UDP-HexNAc and UDP-hexoses were quantified by ultraviolet absorption (A254) and compared with external standards.

**Immunoprecipitation and Immunoblotting**—Subconfluent R28 and L6 muscle cells plated on 60-mm dishes at a density of 4 × 10⁵/cm² were exposed to the indicated concentrations of glucose, mannitol, and glucosamine for 24 h. The cells were deprived of serum for 2 h prior to stimulation with 10 nm insulin for 5 min, 10,000 × g, and perchloric acid. The precipitates were pelleted by centrifugation (5 min, 10,000 × g, 4 °C), and perchloric acid was extracted from the supernatants with 2 volumes of 1:4 trichloroacetic acid (TCA) (Sigma). The aqueous phase was stored at −80 °C and analyzed within 5 days. Nucleotide-linked hexoses and hexosamines were separated and measured by anion exchange high pressure liquid chromatography. UDP-HexNAc and UDP-hexoses were quantified by ultraviolet absorption (A254) and compared with external standards.

**Pulse-Chase Metabolic Labeling**—Subconfluent R28 cells seeded on laminin-coated 100-mm plates were incubated in DMEM containing 5 mM glucose and either 15 mM mannitol or 15 mM glucosamine for 8 h. The cells were washed twice and incubated for 1 h in pre-warmed methionine-free minimal essential medium containing 5 mM glucose plus 15 mM mannitol or glucosamine supplemented with 10 mM dialyzed fetal bovine serum (HyClone) and 25 mM HEPES, pH 7.4. The media were exchanged for 2 ml containing 0.2 mM [³⁵S]methionine. Following incubation at 37 °C for 30 min, the cells were washed and chased for the indicated periods in pre-warmed DMEM plus 10% newborn bovine serum containing 0.2 mM methionine and either 15 mM mannitol or glucosamine. The cultures were washed three times in pre-warmed DMEM plus 10% newborn bovine serum. The supernatants were collected and stored at −80 °C. Nucleotide-linked hexoses and hexosamines were separated and measured by anion exchange high pressure liquid chromatography. UDP-HexNAc and UDP-hexoses were quantified by ultraviolet absorption (A254) and compared with external standards.

**Analysis of Cellular Glucose**—Subconfluent R28 cells were washed three times with ice-cold phosphate-buffered saline and collected with sonicated in 0.5 ml of 1:5 diluted hexokinase (1:2000, Jackson ImmunoResearch). The cells were washed three times with 1 ml of 25 mM HEPES buffer, pH 7.4, containing 10 mM MgCl₂, 1 mM CaCl₂, 2 mM Na₃VO₄, 10 mM sodium pyrophosphate, 10 mM NaF, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, EDTA, EDTA. The cleared supernatant was washed three times with 1 ml of 25 mM HEPES, pH 7.4, 15 mM NaCl, 2 mM EDTA and stored at −80 °C. Nucleotide-linked hexoses and hexosamines were separated and measured by anion exchange high pressure liquid chromatography. UDP-HexNAc and UDP-hexoses were quantified by ultraviolet absorption (A254) and compared with external standards.

**Statistical Analysis**—Statistical comparisons were performed by one-way analysis of variance with post hoc Student–Newman–Keuls multiple comparisons test or by two-tailed unpaired Student’s t test (Instat 2.0; Graphpad Software). (9). Statistical significance was accepted if p < 0.05.
RESULTS

High Glucose and Glucosamine Inhibit the Anti-apoptotic Effect of Insulin on R28 Cells—We have demonstrated previously that insulin can rescue differentiated R28 cells from apoptosis induced by serum withdrawal in a dose-dependent fashion with a maximum effect at 10 nM (9). To test whether high glucose or glucosamine abrogates the rescue effect of insulin, R28 cells were incubated for 24 h in DMEM plus serum containing 5 mM glucose, 20 mM glucose, or increasing concentrations of glucosamine with mannitol as an osmotic control. The cells were then maintained in serum or deprived of serum with or without 10 nM insulin for an additional 24 h. Following the Hoechst staining, the percentage of pyknotic cells in five randomly sampled visual fields per coverslip was calculated. A, a representative picture. Bar indicates 50 μm. B, data represent the mean ± S.E. of five randomly sampled visual fields in n = 3 coverslips. The experiments were repeated three times with reproducible results.

Azaserine Reverses the Inhibitory Effect of High Glucose on Insulin-mediated Neuroprotection—To test whether the increased flux of glucose through the HBP is involved in the attenuated rescue effect of insulin in cells exposed to high glucose, the effect of azaserine, a GFAT inhibitor (12), was investigated. Following the 24-h incubation in the indicated media conditions with or without 0.1 μM azaserine, apoptosis was induced, and the neuroprotective effect of insulin was determined as described above. In addition to the Hoechst staining, immunocytochemistry using CM-1, an antibody specifically recognizing activated caspase-3, was also conducted (9). The ability of insulin to reduce apoptosis was expressed as a ratio of % pyknosis in cells deprived of serum to that in cells deprived of serum and treated with insulin. Data represent the mean ± S.E. of five randomly sampled visual fields in n = 3 coverslips. The experiments were repeated three times with reproducible results.
insulin increased the cell survival only by 1.5-fold regardless of azaserine treatment. Azaserine treatment also normalized the ability of insulin to inactivate caspase-3 in cells incubated in 20 mM glucose (Fig. 2A) but not in cells exposed to glucosamine (data not shown), further supporting the above hypothesis.

High Glucose and Glucosamine Increase UDP-HexNAc in R28 Cells—To confirm whether high glucose or glucosamine leads to increased levels of hexosamine metabolites in R28 cells and, if so, whether GFAT inhibition reverses this effect, intracellular UDP-HexNAc was measured after a 24-h incubation in 5 or 20 mM glucose or 1.5 mM glucosamine, with or without insulin or 0.1 μM azaserine. In the absence of azaserine, the UDP-HexNAc content in cells incubated in 5 mM glucose with insulin decreased by 35% of control (5 mM glucose without insulin and azaserine, 100% = 7.68 ± 1.16 and 10.06 ± 2.05 nmol/mg protein in the absence and presence of azaserine, respectively.) Data represent the mean ± S.D. (n = 3). *, $, and † indicate p < 0.05, 0.001, and 0.0001 versus cells incubated in 5 mM glucose with the same azaserine treatment.

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As reported previously, R28 cells incubated in control media expressed two isoforms of IRβ (125 and 115 kDa) (9), which were also observed in cells exposed to 15 mM mannitol and 20 mM glucosamine (Fig. 4C). In comparison, cells treated with 1.5 and 15 mM glucosamine had an additional isoform of IRα with a lower molecular weight (Fig. 4C). On the other hand, both IRα- and IRβ blots detected a pro-receptor isoform with a molecular mass of 220 kDa in cells treated with control, mannitol, and high glucose media, whereas the electrophoretic mobility of the pro-receptor was increased in a dose-dependent fashion in cells exposed to glucosamine (Fig. 4B). Thus, the abnormal migrating pro-receptor content was also increased in cells treated with 15 mM glucosamine. These results suggest that glucosamine, in particular at higher concentration, may impair IR processing.

High Glucosamine Impairs Processing and Maturation of IR—To confirm whether high glucosamine treatment causes content and tyrosine phosphorylation were quantified. R28 cells were exposed to the indicated combinations of glucose, mannitol, or glucosamine for 4 h, followed by a 2-h serum depletion and a 5-min stimulation with 10 nM insulin. Immunoprecipitated IRβ was phosphorytrosine-blotted and re-probed with IRβ antibody (B), and IRα blotting (C). This experiment is representative of three independent experiments (n = 3). IP, immunoprecipitation; IB, immunoblotting; pro-IR, pro-receptor.

Fig. 3. High glucose and low glucosamine increase the UDP-HexNAc content in R28 cells. R28 cells were incubated in 5 or 20 mM glucose or 1.5 mM glucosamine with or without 10 mM insulin or 0.1 mM azaserine for 24 h. UDP-HexNAc, the end product of the HBP, was measured using anion exchange high pressure liquid chromatography from perchloric acid extracts of R28 cells and normalized to cells incubated in 5 mM glucose without insulin. (100% = 7.68 ± 1.16 and 10.06 ± 2.05 nmol/mg protein in the absence and presence of azaserine, respectively.) Data represent the mean ± S.D. (n = 3). *, $, and † indicate p < 0.05, 0.001, and 0.0001 versus cells incubated in 5 mM glucose with the same azaserine treatment.

Fig. 4. High glucosamine, but not high glucose or low glucosamine, reduces IR autophosphorylation. Following a 24-h incubation in the indicated media conditions and a 2-h serum deprivation, R28 cells were stimulated with 10 nM insulin for 5 min, and cell lysates were subjected to phosphotyrosine (PY) blotting of immunoprecipitated IRβ (A), re-probing with IRβ antibody (B), and IRα blotting (C). This experiment is representative of three independent experiments (n = 3). IP, immunoprecipitation; IB, immunoblotting; pro-IR, pro-receptor.
defective IR processing, pulse-chase metabolic labeling was performed. R28 cells were exposed to 15 mM mannitol or glucosamine for 8 h. Following a 1-h incubation in methionine-free medium, the cells were pulse-radiolabeled with [35S]methionine for 30 min. The cells were then chased for the indicated periods. The cell lysates were immunoprecipitated with IRβ antibody, followed by 7.5% SDS-PAGE and fluorography. The data are representative of two independent experiments (n = 2).

**High Glucose and Glucosamine Do Not Impair Insulin Signaling to PI3K in R28 Cells**—We reported previously (9) that insulin exerts its neuroprotective effect mainly through the PI3K to Akt signaling pathway in R28 cells. To elucidate post-receptor signaling steps where high glucose and low glucosamine perturb the insulin action, we evaluated insulin-stimulated IRS-1 phosphorylation and association of IRS-1 with the p85 subunit of PI3K. Following identical treatments for IR autophosphorylation analysis, R28 cells were harvested. Immunoprecipitated IRS-1 was subjected to phosphoimmunoblotting and p85 blottings. As shown in Fig. 6, 5-min insulin stimulation increased phosphotyrosine content of IRS-1 and p85 content co-immunoprecipitated with IRS-1 in cells exposed to 5 mM glucose, 15 mM mannitol, 20 mM glucose, and 1.5 mM glucosamine (Fig. 6, B and C). IRS-1 protein content was similar among all groups (Fig. 6A). Thus, when normalized to IRS-1 protein content, there was no difference in tyrosine-phosphorylated IRS-1 content or in IRS-1-associated p85 content among cells incubated in control, mannitol, high glucose, and low glucosamine media (n = 3, p = 0.21).

**High Glucose and Low Glucosamine Attenuate Insulin-stimulated Phosphorylation of Akt**—Recent reports (18, 22–24, 28) suggested that high glucose and glucosamine impair insulin-mediated Akt activation in muscle tissues with no significant effect on signaling cascades proximal to Akt. To test whether the inhibitory effect of high glucose and low glucosamine on insulin-mediated anti-apoptosis in R28 cells was associated with the impaired insulin activation of Akt, insulin-stimulated phosphorylation of Akt was quantified. Following a 24-h incubation in the indicated combinations of glucose, mannitol, and glucosamine, R28 cells were stimulated with 10 nM insulin for 30 min. Whole cell lysates were subjected to immunoblotting using an antibody specifically recognizing Akt phosphorylated at Ser473 and one recognizing total Akt (Fig. 7). Without insulin stimulation, Akt phosphorylation was barely detected in any group. Although insulin stimulation increased phospho-Akt content in all groups, cells incubated in 20 mM glucose and in 1.5 mM glucosamine had less phospho-Akt content compared with those incubated in 5 mM glucose or 15 mM mannitol. The total Akt content was similar in all treatment conditions. Thus, when normalized to total protein content, the ratio of phospho-total Akt content after insulin stimulation was reduced to ~75% of the controls in cells exposed to 20 mM glucose or 1.5 mM glucosamine (Fig. 7, without azaserine, p < 0.01). Next, to test whether the increased flux of glucose through the HBP was involved in the reduced insulin activation of Akt, 0.1 μM azaserine was added to the media 24 h prior to insulin stimulation. Insulin-stimulated Akt phosphorylation was increased to the control level in cells exposed to 20 mM glucose and azaserine but was not restored in cells exposed to 1.5 mM glucosamine (Fig. 7, with azaserine). Thus, blocking glucose entry into the HBP reversed the high glucose-induced attenuation of Akt phosphorylation after insulin stimulation.

**High Glucose and Glucosamine Do Not Alter Intracellular ATP Content**—A previous report (46) suggested that intracellular ATP depletion was the major cause of glucosamine-induced insulin resistance in fat cells. To test whether glucosamine reduced ATP content in R28 cells, ATP was measured enzymatically using hexokinase and glucose-6-phosphate dehydrogenase 24 h after incubation in the indicated media conditions. As shown in Fig. 8, ATP content was not significantly different among cells exposed to 5 mM glucose, 15 mM mannitol, and glucosamine.
incubated in 5 mM glucose and correspondingly treated. Each ratio of phospho- to total Akt content after insulin stimulation, expressed as the percentage relative to 5 mM glucose treatment. Each bar represents the mean ± S.E. (n = 4). * indicates p < 0.01 versus cells incubated in 5 mM glucose and correspondingly treated.

Fig. 7. High glucose and low glucosamine attenuate insulin stimulation of Akt phosphorylation in R28 cells. Following a 24-h incubation in the indicated media conditions with or without 0.1 μM azaserine and a 2-h serum deprivation, R28 cells were stimulated with 10 nM insulin for 30 min, and cell lysates were subjected to phospho-Akt (Ser-473) or total Akt immunoblotting (A). B, quantification of relative ratio of phospho- to total Akt content after insulin stimulation, expressed as the percentage relative to 5 mM glucose treatment. Each bar represents the mean ± S.E. (n = 4). * indicates p < 0.01 versus cells incubated in 5 mM glucose and correspondingly treated.

Fig. 8. High glucose and glucosamine do not significantly decrease intracellular ATP content in R28 cells. R28 cells were incubated in the indicated media conditions for 24 h. ATP content was then measured fluorometrically from perchloric acid extracts. Data represent the mean ± S.D. (n = 3).

20 mM glucose, and 1.5 mM glucosamine regardless of insulin stimulation. Cells incubated in 15 mM glucosamine tended to have less ATP content, but the value did not reach statistical significance.

Glucosamine Alters IR Processing but Does Not Induce Apoptosis in L6 Cells—To test whether glucosamine induces aberrant IR processing and apoptosis in other insulin-sensitive cells, L6 cells were treated as described above. IRα and -β immunoblots demonstrated that glucosamine reduced the mature α and β subunits and increased the abnormally migrating pro-receptor isoform in a dose-dependent manner in L6 cells, similar to R28 cells. However, glucosamine did not lead to apoptosis in L6 cells even at the 15 mM concentration (Fig. 9). Thus, glucosamine-induced apoptosis in R28 cells was a cell type-specific event.

DISCUSSION

The present study demonstrated the following findings. 1) High glucose and relatively low concentrations of glucosamine inhibited the ability of insulin to rescue R28 cells, a model of retinal neurons, from apoptosis induced by serum deprivation. 2) The two conditions elevated UDP-HexNAc, the end product of the HBP, in R28 cells. 3) High glucose and glucosamine attenuated Akt phosphorylation after insulin stimulation with no effect on IR autophosphorylation, IRS-1 phosphorylation, and IRS-1/p85 association. 4) These three events were independent of an osmotic stress, because mannitol treatment did not have similar effects. 5) The amidotransferase inhibitor, azaserine, which inhibits GFAT, reversed the above events in cells exposed to high glucose but not to glucosamine, which enters into the HBP distal to GFAT. These lines of evidence strongly suggest that high glucose impairs insulin action as a neurotrophic factor in R28 cells, at least in part, via the excessive flux of glucose through the HBP.

In the present study, 20 mM glucose elevated the UDP-HexNAc content ~2-fold in R28 cells. Previously, we showed (20) that in 3T3-L1 adipocytes, high glucose had a much smaller effect on UDP-HexNAc concentrations, since an 18-h incubation in 25 mM glucose plus 0.6 nM insulin led to only a 30% increase in the UDP-HexNAc content. However, other studies demonstrated that high glucose treatment elevated the nucleotide sugar 2-fold in porcine glomerular mesangial cells and rat-1 fibroblasts (47, 48). An in vivo study indicated that hyperglycemia increased the UDP-HexNAc:UDP-hexose ratio in muscle and to a much lesser extent in liver in rats (41). Thus, the degree to which high glucose increases intracellular UDP-HexNAc depends on the cell type. Interestingly, incubation in 1.5 mM glucosamine increased the hexosamine end product over 15-fold in R28 cells. In a previous report (47) incubation with 7 mM glucosamine gave rise to at most a 4-fold increase in UDP-HexNAc in mesangial cells. However, incubation of 3T3-L1 cells with 0.5 mM glucosamine in the presence of 0.6 mM insulin and 5 mM glucose increased intracellular UDP-HexNAc concentrations ~10-fold. Higher glucosamine concentrations caused no further increase, suggesting limitation of UDP-Hex-
N-acetylglucosamine synthesis at one of two steps beyond hexokinase (30). The present data in R28 cells are consistent with a relatively high capacity HBPs, which may contribute to the susceptibility of retinal neurons to apoptosis.

The impaired insulin activation of Akt without perturbation of the proximal signaling events after exposure to high glucose and/or glucosamine was previously demonstrated in several types of cells and tissues. Heart et al. (18) showed that exposure of 3T3-L1 adipocytes to 50 mM glucose for 6 h attenuated insulin stimulation of Akt activity by 50% with no change in the phosphorylation of IR and IRS-1/2 and with minimal reduction of PI3K activity. In isolated muscles, glucosamine did not alter IR number and IR tyrosine kinase activity (15), and high glucose impaired Akt activation by insulin with PI3K activity being unaffected (22, 23). Similar observations were also reported in Zucker diabetic fatty liver, although the role of the HBPs was not investigated (24). However, in other reports (16, 17, 28) exposure to high glucose or glucosamine affected post-receptor insulin signaling steps proximal to Akt. Singh et al. (28) presented evidence that in rat-1 fibroblasts, 20 mM glucose or 1 mM glucose plus 3 mM glucosamine treatment led to insulin resistance for glycogen synthase activity, which was associated with a reduced ability of insulin to activate PI3K and Akt. In vivo glucosamine infusion studies showed reduced IRS-1 phosphorylation and PI3K activity (16, 17). Although the PI3K activity was not measured in the current study, neither high glucose nor low glucosamine treatment perturbed the insulin signaling cascades proximal to PI3K. Thus, the steps at which high glucose and glucosamine perturb insulin signaling may also depend on cell and tissue types.

No matter which signaling step is initially altered by exposure to high glucose, of importance is that the reduced ability of insulin to stimulate Akt likely renders retinal neurons vulnerable to pro-apoptotic stresses, because insulin exerts its anti-apoptotic effect on neuronal cells, at least in part, through the PI3K/Akt pathway (9, 49). In eyes with diabetic retinopathy, multiple pro-apoptotic factors are induced including oxidative stress, ischemia, and altered glutamate metabolism (50). Therefore, if the current in vitro observations apply to the retina in vivo, hyperglycemia and subsequent activation of the HBP could direct retinal neurons to cell death by impairing the neuroprotective effect of insulin. A 2-fold increase in UDP-N-acetylglucosamine induced by exposure to high glucose had a similar impact on the attenuation of the ability of insulin to rescue R28 cells from apoptosis and stimulate Akt as the 15-fold increase in the nucleotide sugar caused by 1.5 mM glucosamine treatment. Thus, even a modest increase in glucose flux via HBPs may have a critical effect on the function and survival of retinal neurons. Exposure to high glucose may induce several intracellular events, which act synergistically with products of HBPs to block the anti-apoptotic effect of insulin.

The mechanism by which excessive glucose flux through HBPs attenuates insulin-stimulated Akt activity is still unclear. Because UDP-N-acetylglucosamine serves as a substrate for glycoseylation of proteins and lipids (12–14), it is conceivable that glycoseylation of Akt, possibly via O-linked N-acetylglucosamine modification on Ser/Thr residues (51), may be directly affected. Alternatively, hexosamines might regulate the activity of other protein kinases and/or phosphatases.

Another intriguing observation in the present study is that glucosamine at higher concentrations not only inhibited the neuroprotective action of insulin but also induced apoptosis in R28 cells. This glucosamine-induced apoptosis was cell type-specific, because L6 cells were resistant to high glucosamine treatment in the present and in previous studies (52, 53). There are a few possibilities to explain this cytotoxic effect of glucosamine on retinal neurons. The first is the inhibition of N-glycosylation of critical proteins. Tunicamycin, a well known inhibitor of N-linked glycosylation (54), specifically induces apoptotic cell death in neurons such as sympathetic neurons and cerebellar granule cells but not in differentiated PC 12 cells (55, 56), whereas tunicamycin exerts a pro-survival effect in non-neural cells; it can block tumor necrosis factor α-induced apoptosis in hepatocytes (57). Glucosamine, but not other amino sugars such as galactosamine and mannosamine, is also known to inhibit N-glycosylation (54). The defective processing of IR in cells exposed to 15 mM glucosamine in the present study is consistent with previous work (15) in rat-1 fibroblasts overexpressing the human IR. It likely reflects an overall impairment of N-linked glycosylation, because the processing of the IGF-I receptor was also impaired.2 Protein glycosylation is required for neurite elongation, membrane transport of nutrients, and axonal transport (58). Specific alterations in glycosylation of N-linked glycoproteins such as IR and IGF-IR may, therefore, be endogenous signals for the induction of apoptosis in neuronal cells, because glucosamine also affected IR processing in L6 cells with no induction of apoptosis. On the other hand, previous papers (59, 60) indicated that glucosamine as well as tunicamycin preferentially kill tumorigenic cells rather than nontumorigenic cells. Therefore, inhibition of N-glycosylation may have induced apoptosis in R28 cells in part because they are immortalized cells.

Alternatively, glucosamine may inhibit the activity of specific enzymes regulating glycosylation (61). Because the retina highly depends on glycolysis as an energy source, blockade of ATP production from glycolysis would compromise neuronal survival in the retina. Hersko et al. (46) pointed out that ATP depletion might be a mechanism by which glucosamine blocks insulin signaling to glucose transport in 3T3-L1 adipocytes. This seems unlikely, however, in our model, because ATP concentrations were not significantly reduced in R28 cells exposed even to 15 mM glucosamine. Furthermore, a recent report (62) presented evidence against the role of ATP depletion in causing glucosamine on insulin resistance in 3T3-L1 adipocytes.

The third possibility is that accumulated UDP-N-acetylglucosamine may alter the function of critical proteins regulating neuronal viability and functions by O-linked N-acetylglucosamine modification (51). The O-linked glycosylation on serine and threonine residues with N-acetylglucosamine moiety is an important regulatory modification that may have a reciprocal relationship with O-phosphorylation and modulate many biological events in eukaryotes (51).

In vivo, flux through the HBP is highly regulated, in part via allosteric feedback inhibition of GFAT by UDP-N-acetylglucosamine. Because the pro-apoptotic effect in serum-fed R28 cells was only seen after exposure to glucosamine and not after exposure to high glucose, and the former but not the latter caused massive accumulation of UDP-N-acetylglucosamine, it is not clear whether this effect of glucosamine is pharmacological or possibly has its counterpart in the diabetic milieu in the retina. Prolonged exposure to high glucose milieu may cause neuronal cell death. Unfortunately, we could not address this issue in our experimental paradigm, because prolonged culture itself, independently of glucose concentrations, caused conflouency-induced apoptosis in R28 cells (data not shown). However, if this was the case, the activated HBP per se may trigger apoptotic death of neurons in DR independently of impaired insulin action.

In summary, the present study suggests that excessive glucose flux through the HBP may direct retinal neurons to apo-
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ptosis in a bimodal fashion, i.e. via perturbation of insulin action to promote survival, at least in part, mediated by Akt and via induction of apoptosis possibly by altered glycosylation of proteins that maintain cell survival. Diabetes may cause retinal neurodegeneration by the excessive entry of glucose into the HBP.

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