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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 glutamine: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)[1] 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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[1] The abbreviations used are: DR, diabetic retinopathy; HBP, hexosamine biosynthetic pathway; IR, insulin receptor; IRS, insulin receptor substrate; IGF-I, insulin-like growth factor-I; PI3K, phosphoinositide 3-kinase; GFAT, glutamine:fructose-6-phosphate amidotransferase; DMEM, Dulbecco's modified Eagle's medium; UDP-HexNAc, UDP-*N*-acetylhexosamines; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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 stimulation of Akt activity. 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. Trans^{35}S-label metabolic labeling reagents were from ICN Biomedicals (specific activity >1000 Ci/ml; 10 mCi/ml). DMEM with 1000 mg/liter glucose and methionine-free minimal essential medium were purchased from Sigma. Rabbit polyclonal anti-IRα (N-20) and -β (C-19) subunit antibodies were purchased from Santa Cruz Biotechnology. Anti-phospho-Ser^{473} and total Akt antibodies were from Cell Signaling Technology.

Cell Culture—R28 cells were a generous gift from Dr. Gail M. Seigel, State University of New York, Buffalo (7–11). They were grown in DMEM containing 5 mM glucose supplemented with 10% newborn bovine serum (Flow Laboratories) and differentiated to neurons on laminin-coated plates or coverslips with addition of cell-permeable cAMP (Sigma) 24 h prior to the following experiments, as described previously (9). L6 muscle cells (40) were purchased from ATCC and also cultured in DMEM supplemented with 10% newborn bovine serum.

Apoptosis Quantification—R28 and L6 cells seeded at a density of 2×10^5/cm^2 on coverslips were incubated as described above. The media were replaced with DMEM containing the indicated concentrations of glucose, mannitol, and glucosamine for 24 h with or without 0.1 μM azaserine. The cells were maintained in serum, deprived of serum, or deprived of serum and treated with 10 nM insulin for an additional 24 h. The cells were fixed in 1% paraformaldehyde and then incubated with a rabbit polyclonal antibody against activated caspase-3 (1:1000; CM-1, Idun Pharmaceuticals). The secondary antibody was rhodamine red-X-conjugated donkey anti-rabbit (1:2000, Jackson ImmunoResearch). Cells were counter-stained with bisbenzimide Hoechst 33258 (0.5 μg/ml; Sigma) (9). Five visual fields under fluorescence microscope were randomly sampled from each coverslip, and all the cells stained with Hoechst dye in each field were counted. The number of pyknotic cells with condensed or fragmented nuclei was summated in the five sampled regions. The percentage of pyknotic cells per coverslip was then calculated as described (9). In parallel, the percentage of CM-1 immunoreactive cells per coverslip was also measured.

UDP-HexNAc Assay—UDP-HexNAc, the end product of the HBP, was measured as described previously (20, 41). After a 24-h incubation in 5 mM glucose, 20 mM glucose, or 5 mM glucose plus 1.5 mM glucosamine, with or without 10 nM insulin or 0.1 μM azaserine, 100-mm plates of R28 cells were washed once with ice-cold phosphate-buffered saline and homogenized in 0.5 ml of 0.3 M perchloric acid. The precipitates were pelleted by centrifugation (5 min, 10,000 $\times$ *g*, 4 °C), and perchloric acid was extracted from the supernatants with 2 volumes of 1:4 trioctylamine: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 (A_{254}) and compared with external standards.

Immunoprecipitation and Immunoblotting—Subconfluent R28 and L6 cells plated on 60-mm dishes at a density of 4×10^5/cm^2 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 or 30 min and then solubilized in immunoprecipitation buffer (50 mM HEPES, pH 7.3, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM Na_3VO_4, 10 mM sodium pyrophosphate, 10 mM NaF, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, 10% glycerol, 1% Nonidet P-40, and 1 protease inhibitor tablet/10 ml). Protein concentrations were determined with Pierce BCA reagent against bovine serum albumin standard. Tyrosine phosphorylation of IRβ and IRS-1 was determined by immunoprecipitation followed by phosphotyrosine blotting as described previously (9, 33). Whole cell lysates (50 μg) were separated with either 7.5 or 10% SDS-PAGE, transferred, and probed with anti-IRα or phospho-specific or total Akt antibodies (9). Densitometric analysis of enhanced chemofluorescence (Amersham Pharmacia Biotech) immunoblots was performed with ImageQuant (Molecular Dynamics) and ECL immunoblots with NIH Image as described previously (9, 33).

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 plates 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% dialyzed fetal bovine serum (HyClone) and 25 mM HEPES, pH 7.4. The media were exchanged for 2 ml containing 0.2 mCi/ml [^{35}S]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 cells were solubilized in 1 ml of lysis buffer (50 mM HEPES, pH 7.4, 137 mM NaCl, 2 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 1% Triton X-100, 10 mM benzamidine, 1 protease inhibitor tablet/10 ml). Following a 15-min incubation at 4 °C and centrifugation, the supernatant was absorbed for 60 min with 60 μl of protein A-Sepharose. The cleared supernatant was incubated overnight with anti-IRβ antibody at 4 °C. The immune complexes were incubated with protein A-Sepharose for 2 h. The pellets were washed three times with 1 ml of 50 mM HEPES, pH 7.4, 137 mM NaCl, 0.1% Triton X-100, 100 μM phenylmethylsulfonyl fluoride, 0.1% SDS, 1 protease inhibitor tablet/10 ml and then boiled in Laemmli's sample buffer. Proteins were separated by 7.5% SDS-PAGE. Gels were treated with EN3HANCE (PerkinElmer Life Sciences), dried, and subjected to fluorography.

ATP Assay—After a 24-h incubation in 5 mM glucose, 20 mM glucose, or 5 mM glucose plus 1.5 mM glucosamine, with or without 10 nM insulin, 100-mm plates of R28 cells were washed three times with ice-cold phosphate-buffered saline and collected with and sonicated in 0.5 ml of 2% perchloric acid. Following centrifugation (10,000 $\times$ *g*, 2 min, 4 °C), supernatants were neutralized to pH 7.0 with appropriate amounts of 3 M KOH, 0.5 M MOPS, 0.1 M EDTA. To 200 μl of the neutralized cell extract solution, 753 μl of 50 mM HEPES buffer, pH 7.4, containing 10 mM $MgCl_2$ and 5 mM EDTA, 20 μl of 45 mg/ml glucose, 20 μl of 25 mg/ml NADP, 2 μl of glucose-6-P dehydrogenase (≅2.3 units) were added. Following the stabilization of the absorbance at 340 nm, 5 μl of 1:5 diluted hexokinase (≅1.1 units) was added. NADPH, which was generated as a product stoichiometric with ATP, was measured before and after the addition of hexokinase using a Beckman U640 spectrophotometer with analytical wavelength of 340 nm (42). Duplicates were done for each sample, blank, and standard curve measurement. ATP contents were corrected for protein amount in the sample and expressed as nmol/mg protein.

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

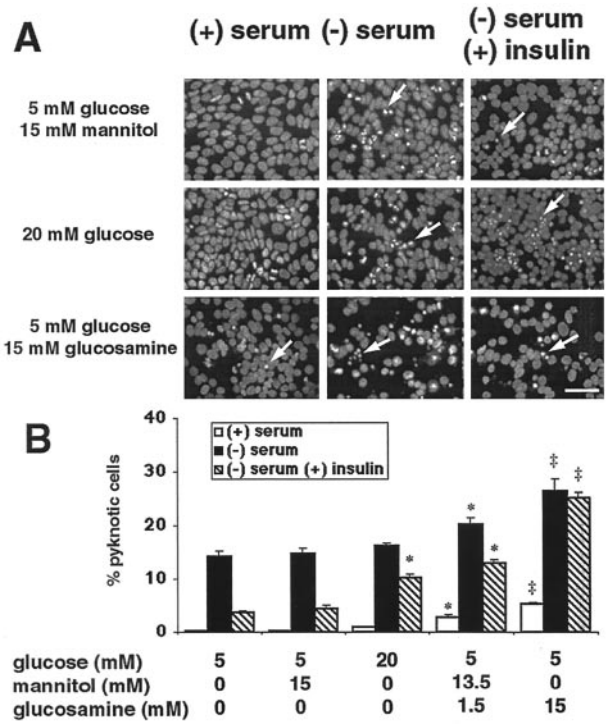

FIG. 1. **High glucose and glucosamine block the anti-apoptotic effect of insulin on R28 cells.** R28 cells on laminin-coated coverslips were incubated in the indicated combinations of glucose, mannitol, and glucosamine for 24 h and then left untreated or deprived of serum with or without 10 nM insulin for an additional 24 h. Following Hoechst 33258 staining, the percentage of pyknotic cells (*arrows*) 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. Insulin rescued R28 cells incubated in 5 mM glucose or 5 mM glucose plus 15 mM mannitol from apoptosis induced by serum withdrawal. Exposure to 20 mM glucose attenuated this neuroprotective effect of insulin. Glucosamine treatment induces apoptosis even in the presence of serum and abrogates the insulin rescue effect in a dose-dependent fashion. * and ‡ indicate $p < 0.05$ and $p < 0.01$ *versus* cells incubated in 5 mM glucose in corresponding serum and insulin treatment conditions, respectively.

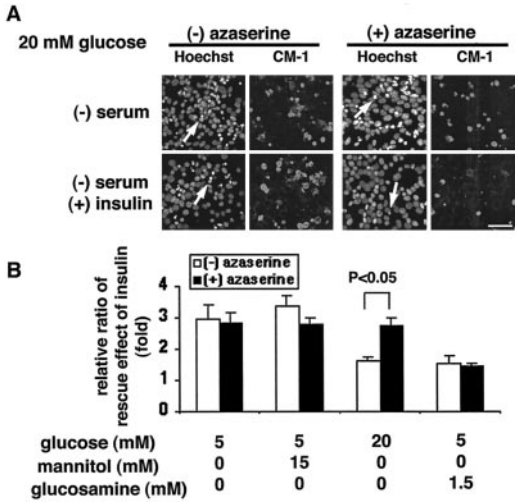

FIG. 2. **Azaserine reverses the inhibitory effect of high glucose but not glucosamine on the neuroprotective effect of insulin.** The contribution of the HBP to the high glucose-induced attenuation of rescue effect of insulin was measured using a GFAT inhibitor, azaserine. Following a 24-h pretreatment with 0.1 μM azaserine, R28 cells were deprived of serum with or without 10 nM insulin for an additional 24 h. The cells were immunostained with CM-1, an antibody recognizing activated caspase-3 and counterstained with Hoechst dye. *A*, a representative picture. Pictures of Hoechst staining and CM-1 immunostaining in each treatment condition were taken from identical fields. *Bar* indicates 50 μm. *B*, the ability of insulin to reduce apoptosis is 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.

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 ($n = 3$) was calculated (Fig. 1). In the presence of serum, less than 1% of cells incubated in 5 mM glucose were pyknotic, whereas serum starvation led to pyknosis in ~15% of cells. Insulin treatment significantly ($p < 0.01$) reduced the number of pyknotic cells to about 8%, which is consistent with our previous observation (9). Exposure to 15 mM mannitol did not show any effects on serum withdrawal-induced apoptosis and insulin rescue of R28 cells. Cells exposed to 20 mM glucose also had very few pyknotic nuclei in the presence of serum, whereas serum deprivation gave rise to pyknosis to a similar degree as controls. However, insulin treatment did not decrease the number of pyknotic cells, suggesting that high glucose had no deleterious effect on cell survival but blocked the ability of insulin to rescue R28 cells from apoptosis. In contrast, glucosamine induced apoptosis even in the presence of serum and augmented the apoptosis induced by serum deprivation in a dose-dependent fashion (Fig. 1). Thus, the total population at counting was substantially less in cells treated with 15 mM glucosamine as compared with other medium conditions (Fig. 1*A*). 1.5 mM glucosamine attenuated the anti-apoptotic effect of insulin to similar a degree as 20 mM glucose, whereas 15 mM glucosamine completely abrogated that effect (Fig. 1*B*).

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 percent pyknosis in cells deprived of serum to that in cells deprived of serum and treated with insulin (Fig. 2*B*). In cells incubated in media containing 5 mM glucose and 5 mM glucose plus 15 mM mannitol, insulin increased cell survival approximately by 3-fold irrespective of the presence or absence of azaserine. Incubation in 20 mM glucose again abrogated the neuroprotective effect of insulin in cells deprived of serum, whereas in the presence of azaserine, 20 mM glucose no longer blocked the rescue effect of insulin (Fig. 2, *A* and *B*). Thus, the HBP was, at least in part, involved in the inhibitory effect of high glucose on insulin-mediated anti-apoptosis. Consistent with this hypothesis, in cells exposed to 1.5 mM glucosamine,

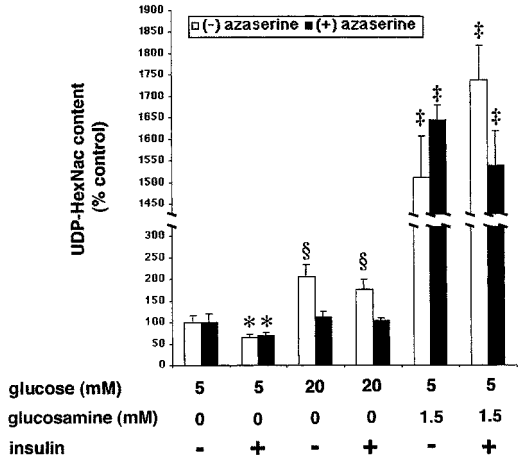

FIG. 3. **High glucose and low glucosamine increase the UDP-HexNAc content in R28 cells.** R28 cells were incubated in 5 or 20 mM glucose or 1.5 mM glucosamine with or without 10 nM insulin or 0.1 μM azaserine for 24 h. UDP-HexNAc, the end product of the HBP, was measured using anion exchange high pressure 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.

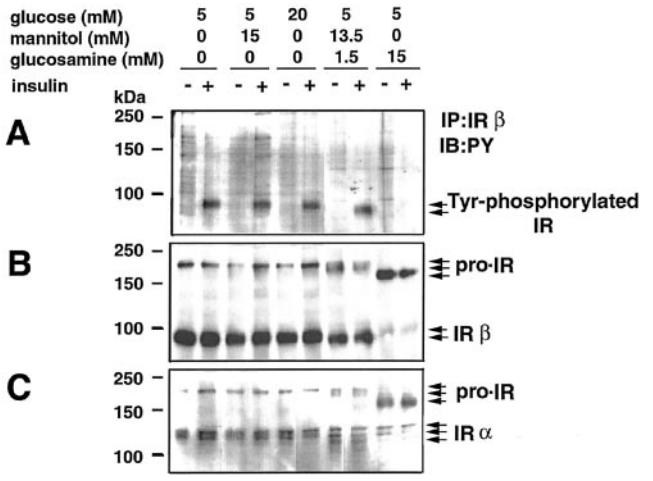

FIG. 4. **High glucosamine, but not high glucose or low glucosamine, reduces IR autophosphorylation.** Following a 24-h incubation in the indicated media conditions and a 2-h serum deprivation, R28 cells were stimulated with 10 nM insulin for 5 min, and cell lysates were subjected to phosphotyrosine (*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.

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. 2*A*) 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 nmol/mg protein; $p < 0.05$), whereas 20 mM glucose treatment significantly increased the content by 205.6 and 173.5% in the absence and presence of insulin, respectively (Fig. 3). In the presence of azaserine, on the other hand, the UDP-HexNAc content in cells incubated in 5 mM glucose with insulin was significantly lower than control (5 mM glucose without insulin and with azaserine, 100% = 10.06 nmol/mg protein; $p < 0.05$), whereas 20 mM glucose did not increase the UDP-HexNAc content irrespective of the presence or absence of insulin. Azaserine alone had no effect on the absolute UDP-HexNAc content in cells incubated in 5 mM glucose (p = 0.154). As expected, incubation in 1.5 mM glucosamine without azaserine increased the UDP-HexNAc content by 1600 and 1800% in the absence and presence of insulin, respectively. Azaserine did not reduce the UDP-HexNAc content in cells irrespective of insulin treatment (Fig. 3). Thus, high glucose and glucosamine increased the hexosamine end product, and GFAT inhibition reversed the UDP-HexNAc content in cells incubated in high glucose but not in low glucosamine. These data also suggest that insulin limits the glucose flux through the HBP at normal glucose concentrations but not when ambient glucose is elevated.

High Glucosamine, but Not High Glucose or Low Glucosamine, Reduces IR Autophosphorylation—To elucidate whether attenuation of insulin action by high glucose and glucosamine occurs at the receptor or at the post-receptor levels, the IR content and tyrosine phosphorylation were quantified. R28 cells were exposed to the indicated combinations of glucose, mannitol, or glucosamine for 24 h, followed by a 2-h serum depletion and a 5-min stimulation with 10 nM insulin. Immunoprecipitated IRβ was phosphotyrosine-blotted and re-probed with IRβ antibody (Fig. 4, *A* and *B*). Whole cell lysates were also blotted for IRα (Fig. 4*C*). Previously we demonstrated that at this concentration insulin specifically activates IR and does not activate IGF-I receptor in R28 cells (9).

Insulin stimulation substantially increased the phosphotyrosine content of IRβ in cells incubated in control media and in those exposed to 20 mM glucose, 15 mM mannitol, or 1.5 mM glucosamine, whereas there was no detectable phosphotyrosine in IRβ in cells exposed to 15 mM glucosamine (Fig. 4*A*). IRβ content was similar among cells treated with 5 mM glucose, 15 mM mannitol, and 20 mM glucose and slightly reduced in cells exposed to 1.5 mM glucosamine (Fig. 4*B*). Thus, when normalized to total IRβ content, there was no difference in insulin-stimulated IR tyrosine phosphorylation among cells incubated in control, mannitol, high glucose, and low glucosamine media (n = 3, p = 0.34). In contrast, cells treated with 15 mM glucosamine expressed only a trace of IRβ (Fig. 4*B*). Thus, the lack of insulin stimulation of IRβ autophosphorylation in cells exposed to 15 mM glucosamine primarily resulted from reduced IRβ content.

As reported previously, R28 cells incubated in control media expressed two isoforms of IRα (125 and 115 kDa) (9), which were also observed in cells exposed to 15 mM mannitol and 20 mM glucose (Fig. 4*C*). In comparison, cells treated with 1.5 and 15 mM glucosamine had an additional isoform of IRα with a lower molecular weight (Fig. 4*C*). On the other hand, both IRα and -β 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. 4, *B* and *C*). In addition, the abnormally 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

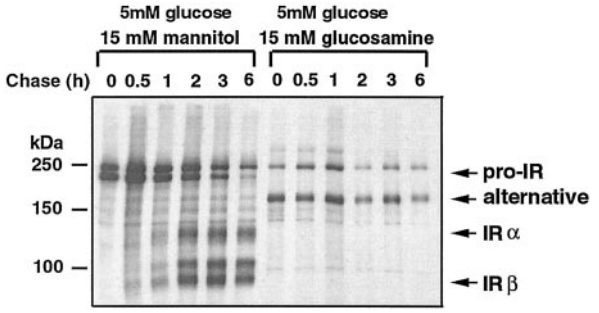

FIG. 5. **Glucosamine impairs IR processing and maturation.** R28 cells were incubated in 15 mM mannitol or 15 mM glucosamine for 8 h. The cells were exposed to methionine-free medium for 1 h, followed by incubation in 200 μCi/ml [^{35}S]methionine for 30 min. The cells were then chased for the indicated 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 ($n = 2$).

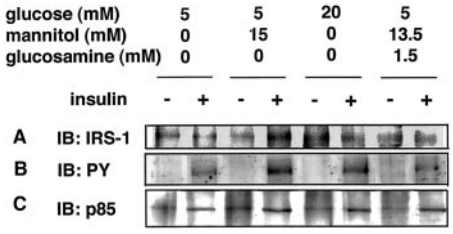

FIG. 6. **High glucose and low glucosamine do not impair IRS-1 phosphorylation and IRS-1/p85 association.** Following a 24-h incubation in the indicated media conditions and a 2-h serum deprivation, R28 cells were stimulated with 10 nM insulin for 5 min, and cell lysates were subjected to IRS-1 (*A*), phosphotyrosine (*PY*) (*B*), and p85 blottings (*C*). This experiment is representative of three independent experiments ($n = 3$). *IB*, immunoblotting.

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 [^{35}S]methionine for 30 min and then chased for the indicated periods. Immunoprecipitated IRβ was subjected to 7.5% SDS-PAGE and fluorography (Fig. 5). The IR precursor was synthesized as a single polypeptide chain and glycosylated with high mannose core oligosaccharides in the endoplasmic reticulum. The ~190-kDa pro-receptor was transported into the Golgi apparatus, where it underwent proteolytic cleavage and oligosaccharide rearrangement to generate the mature IR subunits (43–45). In cells incubated in 15 mM mannitol, one of the major bands at 220 kDa, corresponding to the normal pro-receptor isoform (*pro-IR* in Fig. 5), appeared at the start of chasing and reduced in content over time. Mature IRα and -β subunits corresponding to 125- and 95-kDa bands, respectively, appeared after 0.5 h of chase, increased in content up to 3 h, and started to be reduced after 6 h. There were additional bands with molecular masses of 245 and 105 kDa, both of which could not be detected by immunoblotting with IRβ antibody (Fig. 4*B*). The latter is most likely IGF-IRβ that was co-precipitated with the IRβ, because immunoblotting with the specific antibody detected the IGF-IR β subunit of 105 kDa in IRβ immunoprecipitates (data not shown). Thus IR/IGF-I receptor hybrids are expressed in R28 cells. In comparison, cells exposed to 15 mM glucosamine expressed neither mature IRα nor -β throughout the entire chase periods (Fig. 5). The normal pro-receptor of 220 kDa was not apparent, whereas an alternative isoform of the insulin pro-receptor of 160 kDa appeared, which was also detected by immunoblotting with IRβ antibody (Fig. 4*B*). This alternative form accumulated up to 1 h of chase and decreased thereafter. Another band of 245 kDa was also observed in cells incubated in 15 mM mannitol and could not be detected with the IRβ immunoblotting. Therefore, high glucosamine treatment inhibited normal processing and maturation of the IR and generated alternative products of the pro-receptor.

High Glucose and Low 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 phosphotyrosine 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. 6*A*). 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 Ser^{473} 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- to 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,

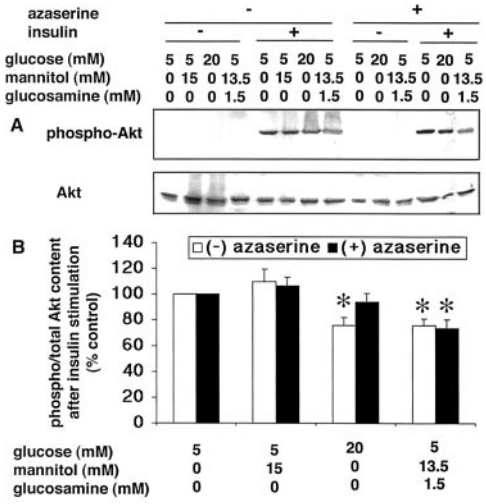

FIG. 7. **High glucose and low glucosamine attenuate insulin stimulation of Akt phosphorylation in R28 cells.** Following a 24-h incubation in the indicated media conditions with or without 0.1 μM azaserine and a 2-h serum deprivation, R28 cells were stimulated with 10 nM insulin for 30 min, and cell 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.

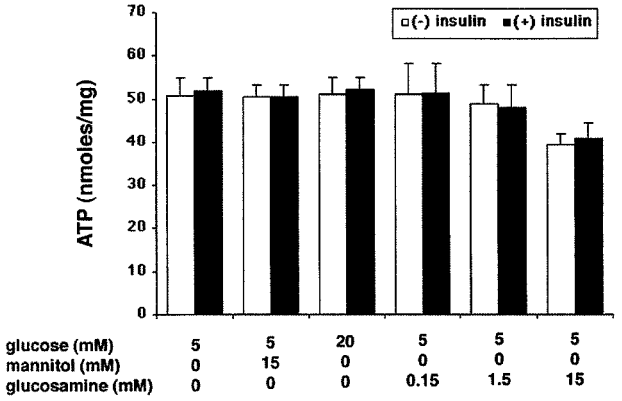

FIG. 8. **High glucose and glucosamine do not significantly decrease intracellular ATP content in R28 cells.** R28 cells were incubated in the indicated media conditions for 24 h. ATP content was then measured fluorometrically from perchloric acid extracts. Data represent the mean ± S.D. ($n = 3$).

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

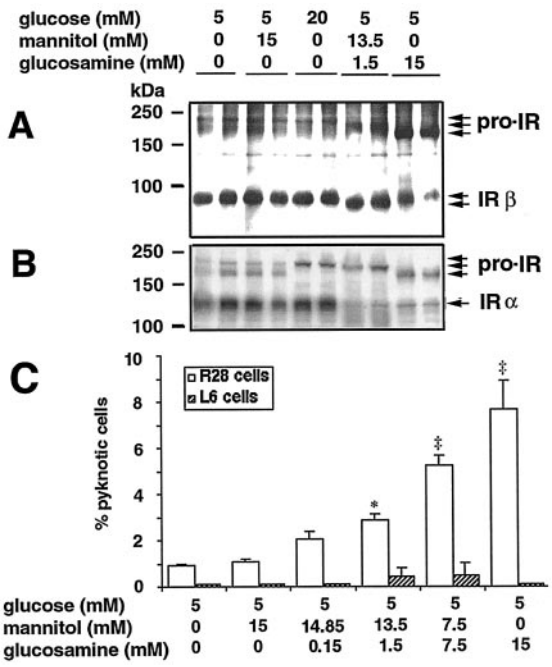

FIG. 9. **Glucosamine affects IR processing but does not induce apoptosis in L6 cells.** Following a 24-h incubation in the indicated media conditions, L6 cell lysates were subjected to IRβ (*A*) or IRα immunoblotting (*B*). *C*, R28 cells and L6 cells were incubated in the indicated concentrations of glucosamine for 24 h, followed by Hoechst staining to determine % pyknosis. 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.

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 nM insulin and 5 mM glucose increased intracellular UDP-HexNAc concentrations ~10-fold. Higher glucosamine concentrations caused no further increase, suggesting limitation of UDP-Hex-

NAc synthesis at one of two steps beyond hexokinase (20). The present data in R28 cells are consistent with a relatively high capacity HBP, 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 glucosamine 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 HBP 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 the 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-HexNAc 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 HBP 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 HBP to block the anti-apoptotic effect of insulin.

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

[2] M. Nakamura and T. W. Gardner, unpublished observations.

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

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