Protein Modification by O-Linked GlcNAc Reduces Angiogenesis by Inhibiting Akt Activity in Endothelial Cells

Bai Luo, Yudi Soesanto, Donald A. McClain

Objective—Glucose flux through the hexosamine biosynthesis pathway (HBP) has been implicated in the development of diabetic vascular complications. O-linked N-acetylglucosamine (O-GlcNAc) modification on protein is the major mechanism mediating the actions of the HBP. Impaired angiogenesis is well-recognized in diabetes; however, the mechanisms are not completely defined. Here, we investigated the role of protein O-GlcNAc modification in angiogenesis.

Methods and Results—In a mouse aortic ring assay, elevated O-GlcNAc levels induced by high-fat diet, streptozotocin-induced diabetes, or in vitro glucosamine treatment were associated with impaired angiogenesis. In cultured human umbilical vein endothelial cells and EA.hy926 endothelial cells, glucosamine increased protein O-GlcNAc modification and inhibited cell migration and capillary-like structure formation. Conversely, removal of O-GlcNAc by adenoviral-mediated overexpression of O-GlcNAcase improved these steps of angiogenesis. Also, high concentrations of glucose reduced capillary-like structure formation of human umbilical vein endothelial cells. Akt was recognized by an O-GlcNAc specific lectin, and glucosamine increased the amounts of Akt protein in these lectin precipitates. Increased glycosylation paralleled reduced Akt activity in endothelial cells.

Conclusion—These results suggest that elevated protein O-GlcNAc modification through the HBP impairs angiogenesis in endothelial cells, possibly by inhibiting Akt signaling. (***Arterioscler Thromb Vasc Biol*. 2008;28:651-657**)

Key Words: hexosamine ■ angiogenesis ■ O-GlcNAc ■ endothelial cells ■ Akt

Vascular complications are the leading cause for morbidity and mortality in diabetic patients, and hyperglycemia is the primary factor in their pathogenesis.[1,2] Angiogenesis, the formation of new blood vessels out of preexisting capillaries, appears to play a pivotal role in the development of diabetic vascular complications.[3,4] Clinical and animal studies have demonstrated abnormally enhanced angiogenesis in the retina, leading to diabetic retinopathy.[3] At the same time, impaired angiogenesis in diabetes often leads to reduced wound healing, exacerbated peripheral limb ischemia, and cardiac mortality through reduced collateral vessel development.[5,6]

See accompanying article on page 608

The effects of hyperglycemia on angiogenesis are not completely understood. Glucose metabolism through the hexosamine biosynthesis pathway (HBP) has been implicated in many of the adverse effects of hyperglycemia, such as insulin resistance in peripheral tissues and diabetic vascular complications.[7–9] In this pathway, a relatively small amount of cellular glucose flux is converted to UDP-N-acetylglucosamine (UDP-GlcNAc) and other amino sugars. The rate-limiting step is catalyzed by glutamine:fructose-6-phosphate amidotransferase (GFA). The levels of the product UDP-GlcNAc are proportional to cellular glucose flux and thus able to serve a nutrient sensing function. UDP-GlcNAc, the chief product of the pathway, is the substrate for O-glycosyltransferase (OGT), which catalyzes the O-linked glycosylation of nuclear and cytosolic proteins with an N-acetylglucosamine (O-GlcNAc) moiety on serine and threonine residues. O-GlcNAc can be removed by β-O-linked N-acetylglucosaminidase (O-GlcNAcase).[7] The O-GlcNAc modification has been observed in numerous proteins and is thought as the main mechanism mediating the nutrient sensing function of the HBP.[9,10] This posttranslational modification modulates protein function in a manner analogous to protein phosphorylation. Indeed, phosphorylation and O-GlcNAc are reciprocal on some well-studied proteins, such as transcription factor Sp1,[11] glycogen synthase,[12] and endothelial nitric oxide synthase (eNOS),[13] further supporting the dynamic significance of the O-GlcNAc modification.

It has been reported that O-GlcNAc levels are elevated in coronary endothelial cells, aortic smooth muscle cells, and atherosclerotic plaques in diabetes, but the physiological consequences remain unknown.[14,15] Because of the central role of angiogenesis in diabetic vascular complications, we examined the effects of protein O-GlcNAc modification on this process. Here, we demonstrate that an increase in O-GlcNAc levels reduces vascular sprouting from mouse aortic rings, migration, and capillary-like tube formation of endothelial cells. Decreased O-GlcNAc, through overexpres-

Original received July 11, 2007; final version accepted December 20, 2007.

From the Division of Endocrinology, University of Utah School of Medicine, Salt Lake City, Utah.

Correspondence to Dr Donald McClain, Division of Endocrinology, University of Utah, Salt Lake City, UT 84132. E-mail donald.mcclain@hsc.utah.edu

sion of O-GlcNAcase, enhances these angiogenic responses. We also found that Akt activity was inhibited by elevated O-GlcNAc levels. These results demonstrate that protein O-GlcNAc modification decreases angiogenesis, in part by inhibiting Akt activity.

Materials and Methods

Reagents

Glucosamine and streptozotocin (STZ) were from Sigma. Phospho-Akt (Ser-473) and Akt antibodies were from Cell Signaling Technology, and anti–O-GlcNAc antibody (RL2) was from ABR Affinity BioReagents. Anti–O-GlcNAc monoclonal IgM antibody (CTD 110.6) was from Dr Gerald Hart (Johns Hopkins University) and O-GlcNAcase antibody was provided by Dr Sidney Whiteheart (University of Kentucky). Succinylated wheat germ agglutinin-agarose (sWGA) was obtained from EY laboratories.

Cell Culture and Adenoviral-Mediated Gene Transfer

Primary human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and grown in endothelial growth medium (EGM) containing 1 g/L glucose according to the manufacturer's instruction. EA.hy926 cells were maintained in low glucose (1 g/L glucose) Dulbecco's modified Eagle's medium (DMEM; Invitrogen). EA.hy926 cells were infected with the adenovirus encoding O-GlcNAcase (a gift from Dr Wolfgang Dillmann, University of California, San Diego) for 2 days. Adenovirus encoding green fluorescent protein (GFP) was used as a control. Preliminary studies revealed that after 48 hours of infection with control GFP, >90% of EA.hy926 cells expressed green fluorescent protein.

Cell Migration Assay

Cell migration was measured using a published cell wounding assay.[16] Endothelial cells were plated at confluence and the monolayer artificially wounded by scraping with a pipette tip. Cells were washed twice with medium and placed in medium containing different concentrations of glucosamine. Phase contrast cell images were taken after healing overnight. The wound healing rates were quantified by measuring the wounding closure using the NIH ImageJ Program.

Capillary-Like Tube Formation Assay

The formation of vascular-like structures by endothelial cells was assessed on growth factor-reduced Matrigel (BD Biosciences), as described.[17] HUVECs or EA.hy926 cells were seeded on Matrigel-coated 24-well plates at 1×10^5 cells per well in 1% FBS medium containing the indicated concentrations of glucosamine and incubated at 37°C for 18 hours. Capillary-like structure formation images were observed using an inverted phase contrast microscope and quantified by the NIH ImageJ Program.

Mouse Aortic Ring Assay

The assay was carried out as described previously.[18] To induce hyperglycemia, C57BL/6J mice (4 to 5 months old, female) were put on high-fat diet for 3 months, or injected intraperitoneally with either saline or on sequential days with 85, 75, and 50 mg of STZ/kg of body weight as described.[19] Blood glucose concentrations were measured before experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Utah. Thoracic aortas were excised from mice, and periadventitial fibroadipose tissues were removed. Aortas were then cut into 1-mm rings, washed in EBM-2 medium (Lonza), and then transferred to 48-well tissue culture plates coated with Matrigel (100 μL per well). The aortic rings were overlaid with an additional 100 μL of Matrigel and allowed to gel for 30 minutes at room temperature. The plates were incubated at 37°C with 0.5 mL EBM-2 medium and media were changed every 2 days. In glucosamine treatment experiments, aortic rings from 7-month-old female C57BL/6J mice were incubated with EBM-2 medium in the presence and absence of glucosamine (5 mmol/L). Aortic rings were examined daily and digital images were taken at day 6 for quantitative analysis of vascular sprouts by the NIH ImageJ Program. Aortic rings and sprouting cells were recovered from Matrigel by Cell Recovery solution (Matrisperse, BD Biosciences), and lysed for Western blot.

Immobilization of Akt With Wheat Germ Agglutinin and Akt Activity Assay

sWGA-agarose was used to precipitate O-GlcNAc modified proteins in EA.hy926 cells as previously described.[20] Akt in sWGA precipitates was detected by Western blot. Akt activity was measured using an Akt assay kit (Cell Signaling).

Statistical Analysis

Data are presented as mean±SE. Differences between treated and control groups were analyzed by 2-tailed Student *t* test, with $P<0.05$ considered statistically significant.

Results

Elevated O-GlcNAc Is Associated With Impaired Angiogenesis in Mouse Aortic Rings

To assess the role of protein O-GlcNAc modification in angiogenesis, aortas from normoglycemic and hyperglycemic mice were used in an aortic ring model for angiogenesis that recapitulates the process of angiogenesis in vitro.[21] Segments of aorta from mice fed normal chow (normoglycemia) or high-fat diet (hyperglycemia) for 3 months were embedded in Matrigel and cultured in endothelial growth medium (EBM-2). Six days after incubation, we observed abundant vascular sprouting from the aortic rings of mice fed normal chow (Figure 1A). As expected, 3 months of high-fat feeding caused insulin resistance and hyperglycemia (fasting blood glucose levels, 133.0±5.5 mg/dL versus 105.9±5.5 mg/dL, $P<0.01$). Aortic rings from high-fat–fed mice had significantly less vascular sprouting as compared with controls (22.5±3.1 versus 45.0±3.4 sprouts per ring, $P<0.01$). Accompanying this, aortic O-GlcNAc levels of high-fat–fed mice were significantly higher than those from normal chow-fed mice (Figure 1B). We next tested in STZ-induced diabetic mice, wherein hyperglycemia was more pronounced (blood glucose level in random fed mice, 337.8±33.5 mg/dL versus 146.4±6.9 mg/dL, $P<0.01$). Consistent with a previous report[19] that STZ-treated mice had increased protein O-GlcNAc modification in fat, we found that STZ treatment caused a 49% increase in total protein O-GlcNAc modification in mice aorta (Figure 1D). Vascular sprouting from aortic rings of STZ-treated mice was significantly reduced (50%) as compared with the rings of control mice (Figure 1C).

To further test the role of O-GlcNAc in angiogenesis, we treated aortic rings with glucosamine. Glucosamine, which enters the HBP downstream of GFA, is more potent than glucose in increasing protein O-GlcNAc in a variety of systems.[8] Treatment of 5 mmol/L glucosamine increased O-GlcNAc levels and decreased vascular sprouting compared with controls (21±1.2 versus 43.2±2.6 sprouts per ring, $P<0.01$; Figure 1E and 1F). These data demonstrate that O-GlcNAc levels correlate with reduced angiogenesis.

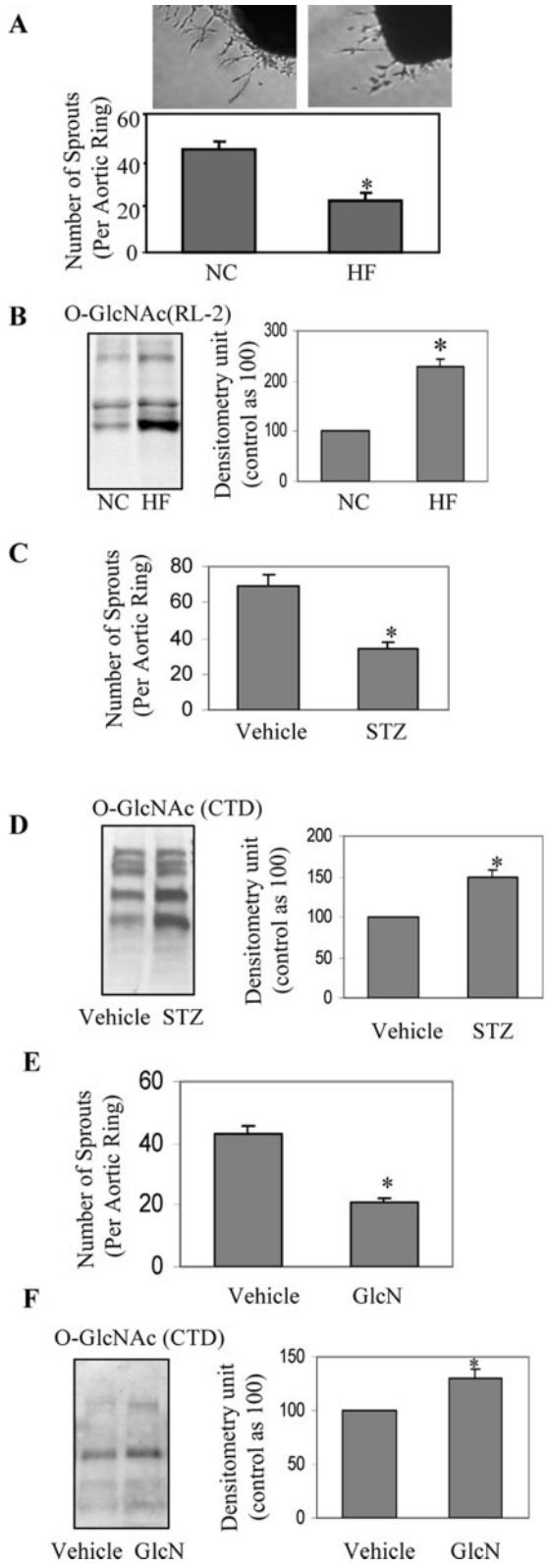


Figure 1. Elevated O-GlcNAc is associated with impaired vascular sprouting from mouse aortic rings. Aortic rings from high fat (HF) diet mice (A, B) or STZ-treated mice (C, D) were embedded in Matrigel. For glucosamine effect, mouse aortic rings from normal chow (NC) mice were treated with glucosamine (GlcN, 5 mmol/L) (E, F). The images of a typical aortic ring experiment and total protein O-GlcNAc modification are also shown. (n≥7, *$P<0.05$ vs controls).

Increased O-GlcNAc Leads to Reduced Migration and Capillary-Like Tube Formation of Endothelial Cells

Angiogenesis involves endothelial cell activation, migration, and capillary-like structure formation.[22] To determine whether O-GlcNAc modification affects the angiogenic potential of endothelial cells, we examined these steps in cultured EA.hy926 cells, derived from the fusion of HUVECs with the human lung carcinoma cell line A549.[23] Like HUVECs, they exhibit the typical endothelial characteristics and are used as an in vitro model for blood vessel–related studies.[24] Glucosamine treatment of EA.hy926 cells significantly increased the levels of cellular O-GlcNAc on protein (Figure 2A). Glucosamine inhibited cell migration in a wound healing assay in a dose dependent manner, with a 31% ($P<0.05$) reduction of the wound healing rate in 2 mmol/L glucosamine as compared with control cells (Figure 2A). We also observed that 2 mmol/L glucosamine inhibited cell migration by 34% ($P<0.01$) in a Boyden chamber assay (data not shown).

Endothelial cells are capable of differentiating in vitro to form capillary-like structures. To determine whether O-GlcNAc affects this angiogenic property of endothelial cells, EA.hy926 cells were cultured in Matrigel-coated wells, and capillary-like tube formation was measured the next day. Glucosamine treatment dramatically inhibited formation of vascular-like structures by 50% and 68%, at 2 mmol/L and 5 mmol/L, respectively (Figure 2B). Because glucosamine may lead to other metabolic changes besides increasing O-GlcNAc levels in cells, we further verified the effect of O-GlcNAc on angiogenesis by treating EA.hy926 cells with O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAc), a pharmacological inhibitor of O-GlcNAcase.[25] PUGNAc also inhibited migration and differentiation of endothelial cells (supplemental Figure, available online at http://atvb.ahajournals.org).

We verified the effect of glucosamine on these angiogenesis steps in primary HUVECs. Glucosamine (5 mmol/L) increased levels of protein O-GlcNAc modification by 97% ($P<0.05$), and inhibited HUVEC cell migration by 47% in the cell wounding assay (Figure 3A). Glucosamine treatment of HUVECs also dramatically inhibited formation of capillary-like structures by 39% ($P<0.01$, Figure 3B). High glucose (20 mmol/L) also increased O-GlcNAc levels in HUVECs and cells treated with high glucose exhibited decreased capillary-like tube formation as compared with cell in low glucose (5 mmol/L; Figure 3C). These data, along with a recent report[26] that high glucose inhibited HUVEC cell migration, suggest that elevated O-GlcNAc levels induced by high HBP flux and hyperglycemia inhibit angiogenic steps in endothelial cells.

O-GlcNAcase Overexpression Increases Migration and Capillary-Like Tube Formation of Endothelial Cells

To further investigate whether O-GlcNAc regulates angiogenic properties of endothelial cells, EA.hy926 cells were infected with a replication-deficient adenovirus encoding O-GlcNAcase. Infection of EA.hy926 cells with the O-GlcNAcase virus in-

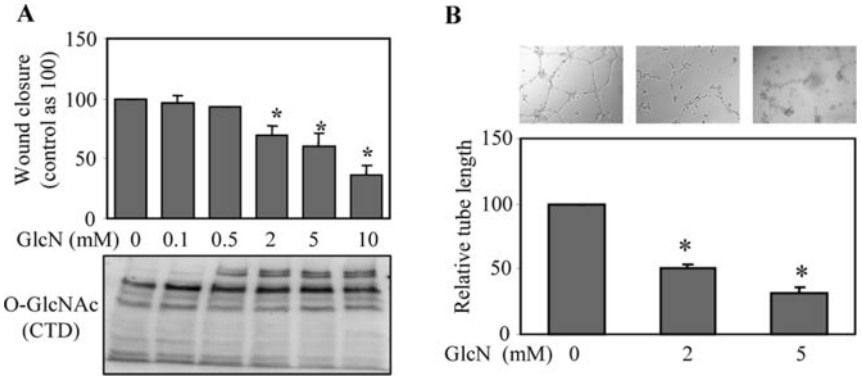


Figure 2. Glucosamine inhibits migration and capillary-like tube formation of EA.hy926 cells. Cell migration (A) and tube formation (B) were measured in EA.hy926 cells treated with or without glucosamine. Total protein O-GlcNAc levels and the images of tube formation in a typical experiment are also shown. (n≥3, *$P<0.05$ vs controls).

creased O-GlcNAcase protein levels and reduced total protein O-GlcNAc modification by 41% ($P<0.01$; Figure 4). EA.hy926 cells infected with the O-GlcNAcase virus exhibited a significantly higher (40%, $P<0.01$) wound healing rate compared with control GFP-infected cells. Overexpression of O-GlcNAcase also increased wound healing rate in glucosamine-treated cells and led to a 68% reversal of the inhibitory effect of glucosamine (data not shown). Additionally, overexpression of O-GlcNAcase increased capillary-like tube formation of EA.hy926 cells by 41% ($P<0.01$). These results indicate that removal of the O-GlcNAc modification in endothelial cells increases their angiogenic properties.

O-GlcNAc Inhibits Akt Activity in Endothelial Cells

Chronically elevated O-GlcNAc levels induce insulin resistance in cultured cell and whole animal models,[10,27] and this may be related to the fact that many signaling molecules of the insulin pathway are known to be modified by O-GlcNAc.[15,28] One of these molecules, Akt, is also known to play a critical role in the regulation of angiogenesis in endothelial cells.[29] We therefore examined the role of Akt in the regulation of angiogenesis by O-GlcNAc. To test whether Akt is O-GlcNAc modified in endothelial cells, we used succinylated wheat germ agglutinin (sWGA), a modified lectin that specifically binds O-GlcNAc, to precipitate the O-GlcNAc modified proteins from EA.hy926 cell lysates. Akt was detected in sWGA precipitates, and glucosamine increased Akt protein levels in sWGA precipitates by 50%

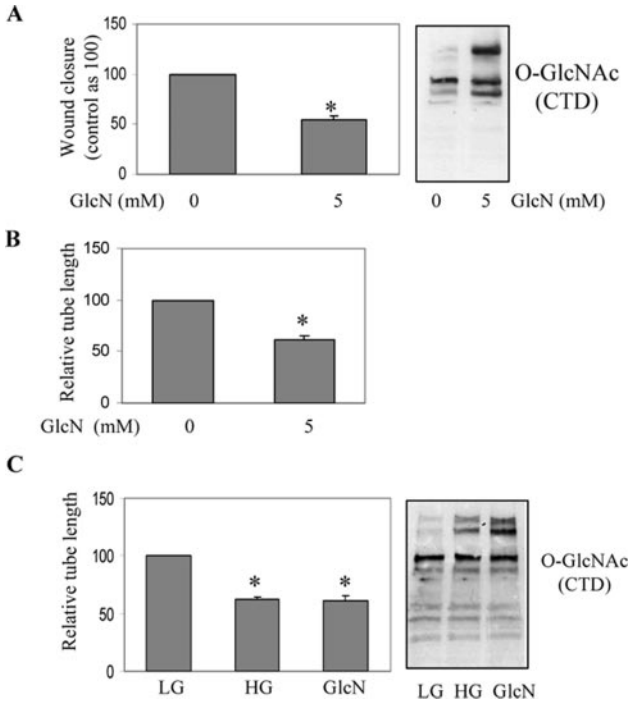


Figure 3. O-GlcNAc inhibits migration and capillary-like tube formation of HUVECs. Cell migration (A) and tube formation (B, C) were measured in HUVECs treated with low glucose (LG, 5 mmol/L), high glucose (HG, 20 mmol/L), or glucosamine (GlcN, 5 mmol/L glucose and 5 mmol/L glucosamine). Total protein O-GlcNAc levels also shown. (n≥3, *$P<0.05$ vs controls).

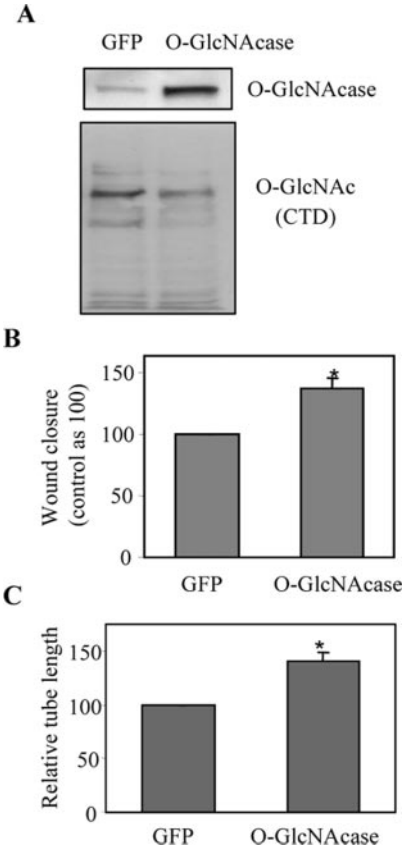


Figure 4. Overexpression of O-GlcNAcase increases migration and capillary-like tube formation of EA.hy926 cells. EA.hy926 cells were infected with adenovirus encoding GFP or O-GlcNAcase for 2 days, and migration (B) and tube formation (C) were measured. O-GlcNAcase protein level and protein O-GlcNAc levels are also shown. (n≥4, *$P<0.05$ vs controls).

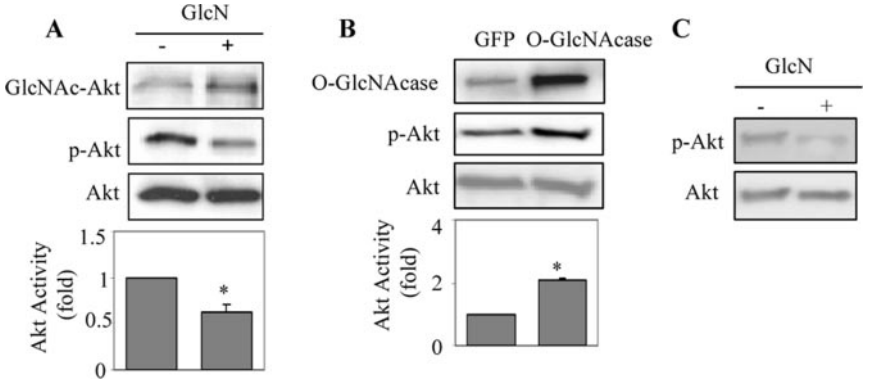


Figure 5. O-GlcNAc inhibits Akt activity in endothelial cells. Akt phosphorylation and activity were measured in lysates from EA.hy926 cells treated with glucosamine (A) or infected with O-GlcNAcase virus (B) or HUVECs treated with glucosamine (C). Glycosylated Akt (GlcNAc-Akt) was detected by sWGA precipitation. (n≥3, *$P<0.05$ vs controls).

($P<0.01$; Figure 5A). Previous studies have shown that phosphorylation of Akt at Ser473 is essential for its activation.[30] Treatment of glucosamine led to reduced Akt phosphorylation and a 38% inhibition of Akt activity with no effect on the levels of total Akt protein. PUGNAc treatment also increased Akt protein in sWGA precipitates while inhibiting Akt activity in EA.hy926 cells (supplemental Figure). Furthermore, removal of O-GlcNAc by O-GlcNAcase overexpression increased Akt phosphorylation and activity in EA.hy926 cells (Figure 5B). These observations suggest that glucosamine and PUGNAc inhibit Akt activity by increasing O-GlcNAc modification of Akt. A recent report showed that high concentrations of glucose also inhibited Akt activity in HUVECs.[26] Consistent with this, we found that glucosamine treatment of HUVECs led to reduced Akt phosphorylation at Ser473 (Figure 5C) and hyperglycemia induced by high-fat diet or STZ treatment also inhibited Akt phosphorylation in mouse aortas (data not shown).

Discussion

Clinical and experimental studies have shown that glucose is the driving force in vascular complications of diabetes. Chronic hyperglycemia dysregulates angiogenic responses in many diabetic complications including retinopathy, coronary artery disease, and peripheral limb ischemia.[2,3,6] The mechanisms underlying this dysregulation remain largely unknown. Endothelial cell proliferation and migration are initial steps critical to the angiogenesis that precede their differentiation into a network of capillary-like structures.[22] Endothelial cells are sensitive to hyperglycemia because of their poor ability to regulate intracellular glucose. A recent report[26] showed that increased glucose concentrations dramatically inhibited proliferation, migration, and differentiation of primary HUVECs into a capillary-like tube structures. A growing body of evidence indicates that some effects of chronically high glucose flux are mediated by the hexosamine biosynthesis pathway (HBP) and increased cellular O-GlcNAc levels.[7,9] Here, we found that glucosamine, by increasing O-GlcNAc levels, significantly inhibited migration and capillary-like tube formation of EA.hy926 and primary HUVECs, and overexpression of O-GlcNAcase largely reversed these inhibitory effects. We also observed that glucosamine inhibited EA.hy926 cell proliferation in a dose-dependent manner (data not shown). Consistent with above results, PUGNAc also inhibited migration and differentiation of endothelial cells, suggesting that increased O-GlcNAc levels are responsible for the reduced angiogenic properties of endothelial cells in vitro. Further supporting a role of O-GlcNAc in angiogenesis, we found that isolated aorta from high-fat–fed or STZ-treated hyperglycemic mice exhibited elevated O-GlcNAc levels and had impaired ability of outgrowth of vessel-like structures in an aortic ring assay. Recently, Shoji et al[31] reported that in vivo angiogenesis measured by a mouse Matrigel plug assay was inhibited in STZ-induced diabetic mice. Taken together, these data suggest that O-GlcNAc might contribute to the impaired angiogenesis in vivo.

The vascular effects of HBP/O-GlcNAc signaling are just beginning to be understood. Hyperglycemia, through the HBP, induces O-GlcNAc modification of transcription factor Sp1 and increases its activity in aortic endothelial cells. This, in turn, enhances transcription of transforming growth factor (TGF)-β1 and plasminogen activator inhibitor (PAI)-1, 2 important factors that regulate vascular function.[11,32] Elevated O-GlcNAc levels with hyperglycemia also led to prolonged calcium transients and cardiac dysfunction in STZ-induced diabetic mouse hearts that could be reversed by overexpression of O-GlcNAcase.[33,34] Elevated O-GlcNAc levels have been observed in coronary endothelial cells and atherosclerotic plaques from diabetic human, and O-GlcNAc modification is increased in rat aortic smooth muscle cells exposed to hyperglycemia.[14,15] A more direct link between increased O-GlcNAc levels and vascular dysfunction has emerged from studies of eNOS.[13,15] eNOS activity in cultured human coronary and bovine endothelial cells was inhibited by hyperglycemia through O-GlcNAc modification and by reciprocal reduction in phosphorylation of Ser-1177. Also, aortas from diabetic animals showed increased eNOS O-GlcNAc modification and decreased eNOS (Ser-1177) phosphorylation, which could contribute to reduced endothelial dependent vasodilatation in diabetic patients. Indeed, Musici et al[35] demonstrated that diabetes-related erectile dysfunction was associated with hyperglycemia-induced eNOS O-GlcNAc modification. Our present study shows that elevated O-GlcNAc induced by high glucose or glucosamine impaired angiogenic processes in endothelial cells and that lowering cellular O-GlcNAc levels by overexpression of O-GlcNAcase improved these processes. These observations support a paradigm where chronically elevated O-GlcNAc levels may represent a common mechanism underlying the adverse effect of hyperglycemia.

O-GlcNAc modification of serine and threonine residues is a highly dynamic posttranslational process that plays a critical role in signal transduction pathways. Like phosphorylation, protein O-GlcNAc modification can impart functional changes in target proteins directly, and also lead to the changes in cellular patterns of gene expression through transcriptional mechanisms.[7] Proteomic analysis reveals more than a hundred O-GlcNAc proteins, including kinases, phosphatases, transcriptional factors and metabolic enzymes; but the functional consequences of this modification remain unknown for most of these proteins.[9] For example, it has been shown that elevated protein O-GlcNAc modification induces insulin resistance in peripheral tissues such as adipocytes and muscle.[27,36] However, it is not clear how and which site of O-GlcNAc modification on proteins leads to impaired insulin signaling. The present study demonstrates that Akt binds to an O-GlcNAc-specific lectin. This binding is responsive to changes in total O-GlcNAc levels in endothelial cells and correlates with reduced Akt activity and phosphorylation, suggesting that O-linked glycosylation plays a role in regulation of Akt activity in endothelial cells. Further supporting this, we found that removal of O-GlcNAc by O-GlcNAcase overexpression increased Akt activity. Consistent with this, Park et al[37] and Gandy et al[38] demonstrated that Akt was O-GlcNAc modified and PUGNAc increased its modification. Importantly, we have identified a physiological consequence of this Akt modification: a reduction of angiogenic response in endothelial cells. Interestingly, phosphatidylinositol 3 kinase (PI3K) protein was not detected in sWGA precipitates and glucosamine did not change PI3K activity in vitro (data not shown). These observations are consistent with a previous report that PUGNAc inhibited Akt phosphorylation in 3T3L1 adipocytes but did not affect insulin receptor β and IRS tyrosine phosphorylation,[27] suggesting that O-GlcNAc regulates Akt activity independent of its upstream protein activators. We have found that Akt is also modified by O-GlcNAc in hepatocytes and mouse liver, and removal of O-GlcNAc increases Akt activity without affecting PI3K activity (data not shown).

Abnormal angiogenesis during diabetes is a well-recognized phenomenon but is somewhat paradoxical: excessive angiogenesis occurs in diabetic retinopathy while the angiogenic response appears to be impaired in macrovascular complications (eg, diabetes-associated coronary artery disease, peripheral limb ischemia).[3,5,6] Angiogenesis is a complex, multistep process subject to multiple hormonal, metabolic, and physical influences (VEGF, hypoxia, level of intracellular glucose, hexosamine flux, redox, etc) that vary from tissue to tissue. It is likely, therefore, that the process of angiogenesis is locally regulated by the continuous interplay of proangiogenic and antiangiogenic signals.[3,4] Indeed, it is apparent that hexosamine pathway could regulate angiogenesis by different mechanisms in different cells. Gardner's group[39] demonstrated that high glucose flux through the HBP induced the retinal neuron apoptosis possibly by altered protein glycosylation, whereas Raman et al[40]showed that hexosamine pathway in vascular smooth muscle cells mediated upregulation of a potent antiangiogenic protein, thrombospondin-1, possibly through O-GlcNAc modification of nuclear proteins. More recently, Brownlee's group demonstrated that O-GlcNAc modification of Sp3 played an important role in the high-glucose–mediated upregulation of angiopoietin-2 gene expression in both microvascular endothelial cells and retinal cells.[41] All of these observations suggest that angiogenesis in diabetes is complex and the HBP is one of many signals that contribute to its regulation and dysregulation.

Acknowledgments

We thank Drs Glendon Parker and David Symons for helpful discussions.

Sources of Funding

This work was supported by the NIH (DK43526 and DK078488) and the Juvenile Diabetes Research Foundation.

Disclosures

None.

References

1. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature*. 2001;414:813–820.
2. Di Mario U, Pugliese G. 15th Golgi lecture: from hyperglycaemia to the dysregulation of vascular remodelling in diabetes. *Diabetologia*. 2001; 44:674–692.
3. Duh E, Aiello LP. Vascular endothelial growth factor and diabetes: the agonist versus antagonist paradox. *Diabetes*. 1999;48:1899–1906.
4. Carmeliet P. Angiogenesis in life, disease and medicine. *Nature*. 2005; 438:932–936.
5. Abaci A, Oguzhan A, Kahraman S, Eryol NK, Unal S, Arinc H, Ergin A. Effect of diabetes mellitus on formation of coronary collateral vessels. *Circulation*. 1999;99:2239–2242.
6. Waltenberger J. Impaired collateral vessel development in diabetes: potential cellular mechanisms and therapeutic implications. *Cardiovasc Res*. 2001;49:554–560.
7. Love DC, Hanover JA. The hexosamine signaling pathway: deciphering the "O-GlcNAc code". *Sci STKE.* 2005;2005:re13.
8. Marshall S, Bacote V, Traxinger RR. Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system. Role of hexosamine biosynthesis in the induction of insulin resistance. *J Biol Chem*. 1991;266:4706–4712.
9. Zachara NE, Hart GW. O-GlcNAc a sensor of cellular state: the role of nucleocytoplasmic glycosylation in modulating cellular function in response to nutrition and stress. *Biochim Biophys Acta*. 2004;1673:13–28.
10. McClain DA, Lubas WA, Cooksey RC, Hazel M, Parker GJ, Love DC, Hanover JA. Altered glycan-dependent signaling induces insulin resistance and hyperleptinemia. *Proc Natl Acad Sci U S A*. 2002;99: 10695–10699.
11. Du XL, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, Wu J, Brownlee M. Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proc Natl Acad Sci U S A*. 2000;97:12222–12226.
12. Parker GJ, Lund KC, Taylor RP, McClain DA. Insulin resistance of glycogen synthase mediated by o-linked N-acetylglucosamine. *J Biol Chem*. 2003;278:10022–10027.
13. Du XL, Edelstein D, Dimmeler S, Ju Q, Sui C, Brownlee M. Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. *J Clin Invest*. 2001;108:1341–1348.
14. Akimoto Y, Kreppel LK, Hirano H, Hart GW. Hyperglycemia and the O-GlcNAc transferase in rat aortic smooth muscle cells: elevated expression and altered patterns of O-GlcNAcylation. *Arch Biochem Biophys*. 2001;389:166–175.
15. Federici M, Menghini R, Mauriello A, Hribal ML, Ferrelli F, Lauro D, Sbraccia P, Spagnoli LG, Sesti G, Lauro R. Insulin-dependent activation of endothelial nitric oxide synthase is impaired by O-linked glycosylation modification of signaling proteins in human coronary endothelial cells. *Circulation*. 2002;106:466–472.

16. Jiang H, Weyrich AS, Zimmerman GA, McIntyre TM. Endothelial cell confluence regulates cyclooxygenase-2 and prostaglandin E2 production that modulate motility. *J Biol Chem*. 2004;279:55905–55913.
17. Nagata D, Mogi M, Walsh K. AMP-activated protein kinase (AMPK) signaling in endothelial cells is essential for angiogenesis in response to hypoxic stress. *J Biol Chem*. 2003;278:31000–31006.
18. Small GR, Hadoke PW, Sharif I, Dover AR, Armour D, Kenyon CJ, Gray GA, Walker BR. Preventing local regeneration of glucocorticoids by 11beta-hydroxysteroid dehydrogenase type 1 enhances angiogenesis. *Proc Natl Acad Sci U S A*. 2005;102:12165–12170.
19. Parker G, Taylor R, Jones D, McClain D. Hyperglycemia and inhibition of glycogen synthase in streptozotocin-treated mice: role of O-linked N-acetylglucosamine. *J Biol Chem*. 2004;279:20636–20642.
20. Luo B, Parker GJ, Cooksey RC, Soesanto Y, Evans M, Jones D, McClain DA. Chronic hexosamine flux stimulates fatty acid oxidation by activating AMP-activated protein kinase in adipocytes. *J Biol Chem*. 2007; 282:7172–7180.
21. Staton CA, Stribbling SM, Tazzyman S, Hughes R, Brown NJ, Lewis CE. Current methods for assaying angiogenesis in vitro and in vivo. *Int J Exp Pathol*. 2004;85:233–248.
22. Griffioen AW, Molema G. Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. *Pharmacol Rev*. 2000;52:237–268.
23. Edgell CJ, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc Natl Acad Sci U S A*. 1983;80:3734–3737.
24. Bouis D, Hospers GA, Meijer C, Molema G, Mulder NH. Endothelium in vitro: a review of human vascular endothelial cell lines for blood vessel-related research. *Angiogenesis*. 2001;4:91–102.
25. Haltiwanger RS, Grove K, Philipsberg GA. Modulation of O-linked N-acetylglucosamine levels on nuclear and cytoplasmic proteins in vivo using the peptide O-GlcNAc-beta-N-acetylglucosaminidase inhibitor O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate. *J Biol Chem*. 1998;273:3611–3617.
26. Yu P, Yu DM, Qi JC, Wang J, Zhang QM, Zhang JY, Tang YZ, Xing QL, Li MZ. [High D-glucose alters PI3K and Akt signaling and leads to endothelial cell migration, proliferation and angiogenesis dysfunction]. *Zhonghua Yi Xue Za Zhi*. 2006;86:3425–3430.
27. Vosseller K, Wells L, Lane MD, Hart GW. Elevated nucleocytoplasmic glycosylation by O-GlcNAc results in insulin resistance associated with defects in Akt activation in 3T3-L1 adipocytes. *Proc Natl Acad Sci U S A*. 2002;99:5313–5318.
28. Zachara NE, Hart GW. O-GlcNAc modification: a nutritional sensor that modulates proteasome function. *Trends Cell Biol*. 2004;14:218–221.
29. Shiojima I, Walsh K. Role of Akt signaling in vascular homeostasis and angiogenesis. *Circ Res*. 2002;90:1243–1250.
30. Alessi DR, Cohen P. Mechanism of activation and function of protein kinase B. *Curr Opin Genet Dev*. 1998;8:55–62.
31. Shoji T, Koyama H, Morioka T, Tanaka S, Kizu A, Motoyama K, Mori K, Fukumoto S, Shioi A, Shimogaito N, Takeuchi M, Yamamoto Y, Yonekura H, Yamamoto H, Nishizawa Y. Receptor for advanced glycation end products is involved in impaired angiogenic response in diabetes. *Diabetes*. 2006;55:2245–2255.
32. Yang X, Su K, Roos MD, Chang Q, Paterson AJ, Kudlow JE. O-linkage of N-acetylglucosamine to Sp1 activation domain inhibits its transcriptional capability. *Proc Natl Acad Sci U S A*. 2001;98:6611–6616.
33. Hu Y, Belke D, Suarez J, Swanson E, Clark R, Hoshijima M, Dillmann WH. Adenovirus-mediated overexpression of O-GlcNAcase improves contractile function in the diabetic heart. *Circ Res*. 2005;96:1006–1013.
34. Clark RJ, McDonough PM, Swanson E, Trost SU, Suzuki M, Fukuda M, Dillmann WH. Diabetes and the accompanying hyperglycemia impairs cardiomyocyte calcium cycling through increased nuclear O-GlcNAcylation. *J Biol Chem*. 2003;278:44230–44237.
35. Musicki B, Kramer MF, Becker RE, Burnett AL. Inactivation of phosphorylated endothelial nitric oxide synthase (Ser-1177) by O-GlcNAc in diabetes-associated erectile dysfunction. *Proc Natl Acad Sci U S A*. 2005; 102:11870–11875.
36. Arias EB, Kim J, Cartee GD. Prolonged incubation in PUGNAc results in increased protein O-Linked glycosylation and insulin resistance in rat skeletal muscle. *Diabetes*. 2004;53:921–930.
37. Park SY, Ryu J, Lee W. O-GlcNAc modification on IRS-1 and Akt2 by PUGNAc inhibits their phosphorylation and induces insulin resistance in rat primary adipocytes. *Exp Mol Med*. 2005;37:220–229.
38. Gandy JC, Rountree AE, Bijur GN. Akt1 is dynamically modified with O-GlcNAc following treatments with PUGNAc and insulin-like growth factor-1. *FEBS Lett*. 2006;580:3051–3058.
39. Nakamura M, Barber AJ, Antonetti DA, LaNoue KF, Robinson KA, Buse MG, Gardner TW. Excessive hexosamines block the neuroprotective effect of insulin and induce apoptosis in retinal neurons. *J Biol Chem*. 2001;276:43748–43755.
40. Raman P, Krukovets I, Marinic TE, Bornstein P, Stenina OI. Glycosylation mediates up-regulation of a potent antiangiogenic and proatherogenic protein, thrombospondin-1, by glucose in vascular smooth muscle cells. *J Biol Chem*. 2007;282:5704–5714.
41. Yao D, Taguchi T, Matsumura T, Pestell R, Edelstein D, Giardino I, Suske G, Rabbani N, Thornalley PJ, Sarthy VP, Hammes H-P, Brownlee M. High glucose increases angiopoietin-2 transcription in microvascular endothelial cells through methylglyoxal modification of mSin3A. *J Biol Chem*. 2007;282:31038–31045.

Arteriosclerosis, Thrombosis, and Vascular Biology

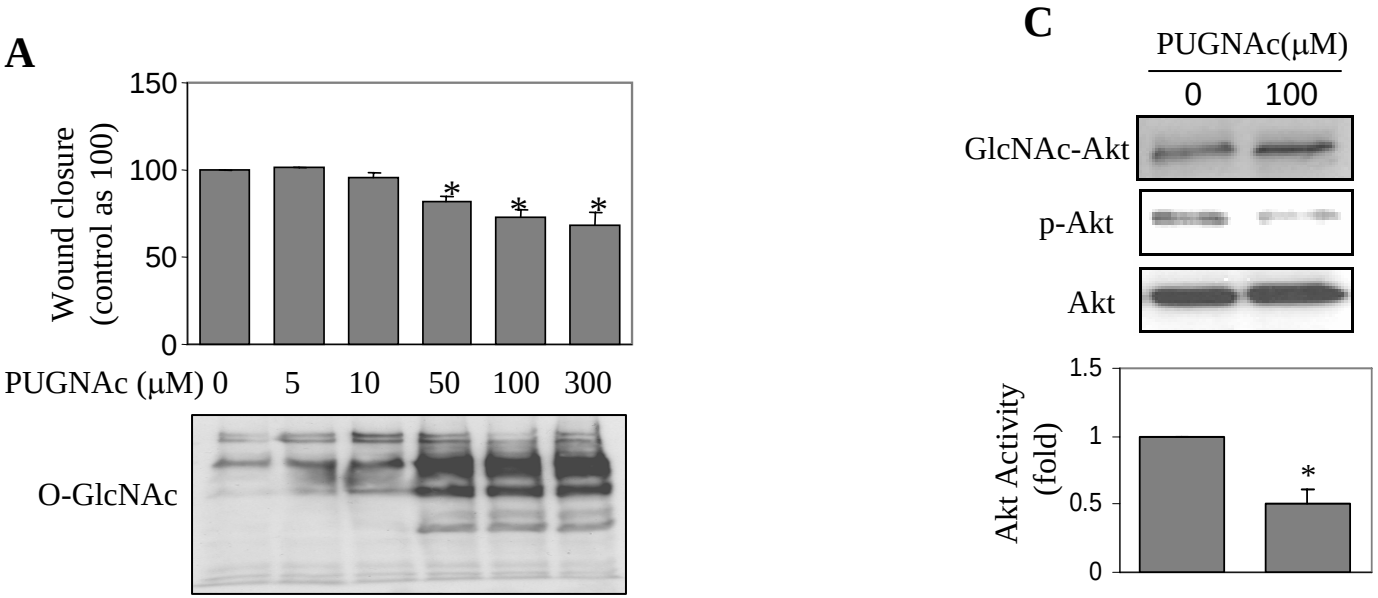
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Protein Modification by O-Linked GlcNAc Reduces Angiogenesis by Inhibiting Akt Activity in Endothelial Cells

Bai Luo, Yudi Soesanto and Donald A. McClain

Arterioscler Thromb Vasc Biol. 2008;28:651-657; originally published online January 3, 2008;
doi: 10.1161/ATVBAHA.107.159533
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636



Supplemental Figure. PUGNAc inhibits migration, capillary-like tube formation and Akt activity in EAhy.926 cells. EA.hy926 cell migration and differentiation were measured in a cell wounding assay (A) and a capillary-like structure formation assay on Matrigel (B) as described in Materials and Methods. EA.hy926 cells were pretreated with PUGNAc at the various concentrations for 2 days before experiments. (C) EAhy.926 cell extracts were immunoblotted with phospho-Akt (Ser473) and total Akt antibodies. To identify the Akt glycosylation (GlcNAc-Akt), cell lysates were precipitated with sWGA agarose followed by immunoblotting with anti-Akt. To measure Akt activity, cell lysates were immunoprecipitated with Akt antibody and incubated with GSK3 fusion protein substrate. The reaction mixture was immunoblotted with a GSK3 phospho-specific antibody. Results are shown as mean ± S.E. of three or four independent experiments. * $p<0.05$ vs. control.