Insulin-Dependent Activation of Endothelial Nitric Oxide Synthase Is Impaired by O-Linked Glycosylation Modification of Signaling Proteins in Human Coronary Endothelial Cells

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Background—Hyperglycemia impairs functional properties of cytosolic and nuclear proteins via O-linked glycosylation modification (O-GlcNAcylation). We studied the effects of O-GlcNAcylation on insulin signaling in human coronary artery endothelial cells.

Methods and Results—O-GlcNAcylation impaired the metabolic branch of insulin signaling, ie, insulin receptor (IR) activation of the IR substrate (IRS)/phosphatidylinositol 3-kinase (PI3-K)/Akt, whereas it enhanced the mitogenic branch, ie, ERK-1/2 and p38 (mitogen-activated protein kinase). Both in vivo and in vitro phosphorylation of endothelial nitric oxide synthase (eNOS) by Akt were reduced by hyperglycemia and hexosamine activation. Insulin-induced eNOS activity in vivo was reduced by hyperglycemia and hexosamine activation, which was coupled to increased activation and expression of matrix metalloproteinase-2 and -9; these phenomena were reversed by inhibition of the hexosamine pathway. Finally, carotid plaques from type 2 diabetic patients showed increased endothelial O-GlcNAcylation with respect to nondiabetics.

Conclusions—Our data show that hyperglycemia, through the hexosamine pathway, impairs activation of the IR/IRS/PI3-K/Akt pathway, resulting in deregulation of eNOS activity. (Circulation. 2002;106:466-472.)

Key Words: atherosclerosis ■ diabetes mellitus ■ endothelium ■ insulin ■ metalloproteinases

Endothelial dysfunction is an early step in the pathogenesis of atherosclerosis and is a feature of insulin-resistant states such as diabetes, obesity, and hypertension. Insulin induces vasodilation by modulating endothelial nitric oxide synthase (eNOS) activity and expression through activation of the insulin receptor (IR)/IR substrate (IRS)-1 and IRS-2/phosphatidylinositol 3-kinase (PI3-K)/protein kinase B/Akt signaling cascade.

In type 2 diabetes, insulin resistance is associated with impaired activation of the IR/IRS/PI3-K pathway but normal activation of the Ras/mitogen-activated protein kinase (MAPK) pathway.

Activation of the hexosamine pathway by hyperglycemia results in O-linked glycosylation modification (O-GlcNAcylation) of Ser/Thr residues of insulin-signaling proteins, which affects receptor assembly and PI3-K activation. It was recently observed that hyperglycemia has toxic effects on endothelial cells through induction of mitochondrial superoxide. Superoxide was found to shift glucose metabolism through the hexosamine pathway, and increased glucosamine production was proposed to contribute to endothelial cell deregulation. Endothelial production of NO plays an important role in preventing vascular disease through regulation of thrombosis, inflammation, vascular tone, and remodeling. NO has been shown to inhibit smooth muscle cell migration and proliferation, as well as synthesis and secretion of extracellular matrix proteins. A key role in this process is played by matrix metalloproteinases (MMPs), which degrade extracellular matrix proteins and are counterbalanced by tissue inhibitors of metalloproteinases (TIMPs). An altered balance between MMPs and TIMPs has been implicated in many conditions related to atherosclerosis, such as plaque instability and restenosis after angioplasty, observed in diabetic patients. These observations prompted us to test the hypothesis that hyperglycemia via hexosamine activation induces selective insulin resistance in human coronary artery endothelial cells (HCAECs) by affecting key insulin-signaling molecules.

Methods

Materials

Antibodies and reagents used were as follows: anti-phosphotyrosine, anti-IRβ, anti-IGF-1Rβ, anti-IRS-1, anti-IRS-2, anti-p85, anti-
ser1177-eNOS, and plasmid pc-DNA3 encoding constitutively active Akt (UBI, Upstate, Lake Placid, NY); anti-Akt, anti-Ser473-Akt, anti-Ser259-c-Raf-1, anti-c-Raf-1, anti-ERK-1/2, and anti-phospho-ERK-1/2 (Cell Signaling Technology, Waltham, Mass); anti-human eNOS (Transduction Laboratories, Franklin Lakes, NJ); anti-O-linked glyco-
sylation RL-2 (Affinity Bioreagents, Golden, Colo); anti-MMP-2, anti-
MMP-9, and PD098059 (Calbiochem, San Diego, Calif); and anti-
TIMP-1, anti-TIMP-2, and anti-TIMP-3 (Chemicon, Temeacula, Calif).
All other chemicals were from Sigma (St Louis, Mo).

Cell Culture
HCAECs were grown according to the manufacturer’s instructions
(Clonetics Corp, Brescia, Italy). Dose-response experiments
were performed at different glucose (5.5, 10, 20, and 30 mmol/L) and
glucosamine (0, 1.5, 7.5, and 15 mmol/L)18,19 concentrations for 72
hours to compare O-GlcNAcylation of immunoprecipitated IRSs and
p85. Intracellular ATP levels were measured by an ATP assay kit
(Calbiochem) as indicated by the manufacturer. HCAECs from
passages 3 to 6 were cultured for 72 hours in the presence of
5.5 mmol/L glucose, 20 mmol/L glucose (high glucose; HG),
7.5 mmol/L glucosamine (GLN), and 5.5 mmol/L glucose plus
1.5 mmol/L manitol. Azaserine (10 μmol/L) and PD098059 (100
μmol/L) were used to block the hexosamine pathway in 20 mmol/L
glucose (HG plus azaserine) or MAPK activity (HG plus
PD098059).

IRS-1, IRS-2, Akt, ERK-1/2, p38, and c-Raf-1 Phosphorylation and Anti-Phosphotyrosine–
Associated PI3-K Activity
At the end of treatments, HCAECs were serum starved for 16 hours and incubated in the presence or absence of 50 mmol/L insulin for 10
minutes. Cells lysis, immunoprecipitations, Western blotting, and
PI3-K assays were performed as described previously.20 Membranes
blotted with anti-phosphotyrosine or phoshoactive antibodies were
stripped and reprobed with primary antibodies to verify expression
levels. Protein phosphorylation levels were normalized to the matching
densitometric values of nonphosphorylated proteins. Autoradiograms
were quantified with a FluorImager (Bio-Rad).

In Vitro Akt Kinase Assay on
Immunoprecipitated eNOS
eNOS immunoprecipitates were obtained from cells cultured as
described above. Active Akt kinase was obtained by immunopre-
cipitation of overexpressed Myc-tagged constitutively active Akt
from transfected HEK293.21,22 The immunoprecipitates were com-
bined and incubated in Akt kinase assay buffer, and the reaction was
terminated as described previously.20–22 Then, samples were sub-
jected to 8% SDS-PAGE and analyzed by a phosphorimager (Bio-
Rad). eNOS content, phosphorylation on Ser1177, and
O-GlcNAcylation were verified by Western blotting with specific
antibodies.

eNOS Activity
The amount of NOS activity produced by HCAECs was measured
by the NOS Detect assay kit (Stratagene) through the conversion
of [14C]-arginine to [14C]-citrulline, according to the manufacturer’s
instructions.6 Data were normalized by the amount of protein and
reaction time.

SDS-PAGE Zymography and Reverse Zymography
MMP activity could be quantitatively estimated by a zymography
assay as described previously.23 In this method, proteins that had
gelatinolytic activity were detected on electrophoretic migration on
the basis of their capacity to digest the gelatin substrate incorporated
into gels. To this end, cultured supernatants were concentrated,
mixed with an equal volume of nonreducing buffer, and electropho-
resed in discontinuous 10% SDS-PAGE gel containing 1 mg/mL
gelatin (Novex). Gels were incubated in Novex zymogram renaturation
buffer and equilibrated in Novex zymogram developing buffer
for 18 hours at 37°C. Gels were stained with Coomassie blue R-250
followed by destaining in 5% methanol and 7% acetic acid. MMPs
appear as white bands on a blue background. Reverse zymography
was performed to measure TIMP activity as follows: samples were
electrophoresed in discontinuous 12% SDS-PAGE gels containing 1
mg/mL gelatin and conditioned medium from baby hamster kidney
(BHK) cells expressing gelatinase A. The gels were washed and
incubated for 24 hours at 37°C in regenerating buffer (50 mmol/L
Tris/HCl, pH 7.5, and 5 mmol/L CaCl2). Under these conditions,
TIMPs inhibit gelatin digestion by activated MMPs, producing dark
blue bands against a lighter background. Density of bands was
quantified by the National Institutes of Health IMAGE program,
version 1.6. A single value was derived for MMP-9 and for MMP-2
by adding the bands associated with both pro and activated forms.

Tissue Immunohistochemistry
Formaldehyde-fixed and paraffin-embedded carotid and ovary sec-
tions were immunostained as described previously24 with RL2
antibody according to the manufacturer’s instructions. Negative
controls were performed that omitted the primary antibody. Positive
signals in the cap plaque were measured by the National Institutes
of Health IMAGE program, version 1.6.

Statistical Analysis
Results are reported as percent increase with respect to control.
Statistical analyses were performed with 1-way ANOVA or Stu-
dent’s t test as indicated. Values of P<0.05 were considered
statistically significant.

Results
Dose-Dependent Effects of Glucose and
Glucosamine on HCAECs
O-GlcNAcylation levels of IRSs and p85 proteins were
assessed on exposure to different glucose and glucosamine
concentrations. Results showed that IRS-1, IRS-2, and p85
O-GlcNAcylation levels were increased by 400%, 175%, and
170%, respectively, at 10 mmol/L glucose or 1.5 mmol/L
and by 600%, 210%, and 200%, respectively, at
20 mmol/L glucose or 7.5 mmol/L glucosamine. No further
increase in IRS and p85 protein O-GlcNAcylation was
observed by increasing concentrations up to 30 mmol/L
or 15 mmol/L glucosamine (Figure 1). Intracellular
ATP levels were similar in cells cultured under the different
conditions with respect to control cells (data not shown).
Subsequent experiments were thus performed that studied
cells cultured in glucose 5.5 mmol/L (control) versus HG or
GLN. A similar glucosamine concentration was used in human
cells and porcine mesangioblasts to mimic the chronic
hyperglycemia-induced hyperactivity of the hexosamine
pathway that is characteristic of type 2 diabetes.11,18,19

HG and GLN Impair Insulin Signaling in HCAECs
Incubation of HCAECs with HG and GLN resulted in 50%
reduction in insulin-stimulated IR tyrosine phosphoryla-
tion with respect to control cells (P<0.001; n=3; Figure
2a). No insulin-induced IGF-1R-β (insulin-like growth
factor-1 receptor-β) phosphorylation was observed in these
conditions (data not shown). Insulin-induced tyrosine
phosphorylation of IRS-1 was reduced by 56% and 63% in
HG and GLN, respectively (P<0.0001; n=3). Insulin-
stimulated coprecipitation of p85 with IRS-1 was reduced
by 20% and 35% in HG and GLN, respectively \((P<0.001; n=3)\). Insulin-stimulated IRS-1–associated PI3-K activity was reduced by 55% and 45% in HG and GLN, respectively \((P<0.001; n=3; \text{Figure 2b})\). Similarly, insulin-induced IRS-2 tyrosine phosphorylation, p85 coprecipitation with IRS-2, and IRS-2–associated PI3-K activity were significantly reduced in HG and GLN HCAECs with respect to control HCAECs (Figure 2c). No differences were observed in IR, IRS-1, IRS-2 (Figure 2), or p85 (data not shown) levels under basal or activated conditions. PI3-K activity associated with phosphotyrosine immuno-precipitates was reduced by 50% and 28% in HG and GLN HCAECs, respectively \((P<0.001; n=3; \text{Figure 3a})\). Insulin-stimulated Akt phosphorylation was reduced by 30% and 28% in HG and GLN HCAECs, respectively \((P<0.0005; n=4)\). Insulin-stimulated c-Raf-1 phosphorylation was reduced by 61% and 47% in HG and GLN HCAECs, respectively \((P<0.0004; n=3)\). Insulin-induced ERK-1/2 phosphorylation was increased by 49% and 53% in HG and GLN HCAECs, respectively \((P<0.0001; n=3)\). Insulin-stimulated p38 phosphorylation was increased by 275% and 118% in HG and GLN HCAECs, respectively \((P<0.0001; n=3; \text{Figure 3, b through e})\). No differences were observed in Akt, c-RAF-1, ERK-1/2, and p38 total protein levels under basal or activated conditions (Figure 3, b through e). No differences in insulin-stimulated signaling pathways were observed in control and mannitol conditions (data not shown).

**Figure 1.** O-GlcNAcylation levels of IRS and p85 proteins at different glucose and glucosamine concentrations. Dose-response experiments were performed at different glucose (5.5, 10, 20, and 30 mmol/L) and glucosamine (0, 1.5, 7.5, and 15 mmol/L) concentrations to compare O-GlcNAcylation levels in IRS-1 (left), IRS-2 (middle), and p85 (right). IP indicates ImmunoPrecipitation; WB, Western Blot.

**Figure 2.** HG and GLN impair insulin activation of IR/IRS/PI3-K pathway. Effects of insulin stimulation of HCAECs cultured in presence of 5.5 mmol/L glucose (Ctrl), 20 mmol/L glucose (HG), or 7.5 mmol/L glucosamine (GLN) on (a) IR-β phosphorylation; (b) IRS-1 phosphorylation, IRS-1-coprecipitated p85, and IRS-1–associated PI3-K activity; and (c) IRS-2 phosphorylation, IRS-2-coprecipitated p85, and IRS-2–associated PI3-K activity, \(n=3\). ***\(P<0.0001\), **\(P<0.001\), *\(P<0.01\) in insulin-stimulated HG or GLN compared with insulin-stimulated control, by ANOVA. a.u. indicates arbitrary units; PY, phosphotyrosine; IP, ImmunoPrecipitation; and WB, Western Blot.
eNOS Phosphorylation Is Reduced by HG and GLN

Analysis of eNOS protein by the YinOYang prediction server allowed identification of several O-GlcNAcylation sites around the Ser1177 residue (Figure 4a). eNOS O-GlcNAcylation was increased by 294% in HG HCAECs, whereas inhibition of hexosamine biosynthesis by the glutamine:fructose-6-phosphate aminotransferase (GFAT) inhibitor azaserine reversed the HG effect (P < 0.0001; n = 4; Figure 4b). In the in vitro kinase assay, incorporation into eNOS induced by constitutively active Akt was reduced by 46% and 83% in eNOS immunoprecipitated from HG and GLN HCAECs, respectively (P < 0.0001; n = 4) and was reversed by azaserine treatment (Figure 4c). Immunoblotting with anti-pSer1177eNOS reproduced results observed with kinase assays (data not shown and Figure 4c). There was an inverse relation between O-GlcNAcylation extent and phosphorylation status (Figure 4c). Insulin-stimulated pSer1177eNOS phosphorylation was reduced by 37% and 42% in HG and GLN HCAECs, respectively (P < 0.012; n = 4; Figure 4d). Insulin-stimulated eNOS activity was reduced by 27% and 22% in HG and GLN HCAECs, respectively (P < 0.001; n = 4); no differences were observed in basal eNOS activity (Figure 4e).

MMP-2 and MMP-9 Activity Is Increased in HCAECs Treated by HG and GLN

NO produced by eNOS inhibits MMP-2 and MMP-9 activity. 

![Figure 3. HG and GLN impair metabolic branch of insulin signaling but not mitochondrial branch. Effects of insulin stimulation of HCAECs cultured in presence of 5.5 mmol/L glucose (Ctrl), 20 mmol/L glucose (HG), or 7.5 mmol/L glucosamine (GLN) on (a) immunoprecipitated phosphotyrosine-associated PI3-K activity; (b) Ser473Akt phosphorylation; (c) Ser259c-Raf-1 phosphorylation; (d) ERK-1/2 phosphorylation; and (e) p38 phosphorylation. Significant differences between insulin-stimulated HG and GLN compared with insulin-stimulated control are indicated. n = 3. ***P < 0.0001, **P < 0.001, §P < 0.0005, P < 0.0004, by ANOVA. PY and py indicate phosphotyrosine; tyr, tyrosine; phospho, phosphorylation; and IP, ImmunoPrecipitation.]

![Figure 4. HG and GLN impair insulin-stimulated eNOS activity. O-GlcNAcylation. a, Predictive analysis of human eNOS O-GlcNAcylation sites based on YinOYang prediction server. b, Effects of HG in presence or absence of 10 μmol/L azaserine on eNOS O-GlcNAcylation. c, Effects of Akt stimulation on 32P incorporation into eNOS in in vitro kinase assay (panel 1), on pSer1177eNOS phosphorylation (panel 2); O-GlcNAcylation (panel 3) and protein (panel 4) levels of eNOS. d, Effects of insulin on pSer1177eNOS phosphorylation in HG and GLN HCAECs (n = 4; P < 0.012). e, Effects of insulin on eNOS activity in HG and GLN HCAECs. Significant differences among insulin-stimulated HG and GLN compared with insulin-stimulated control are indicated. n = 4. ***P < 0.0001, **P < 0.001, *P < 0.012, by ANOVA.]

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72% in HG and GLN HCAECs, respectively (P<0.01; n=3). MMP-2 activity was increased by 87% and 119% in HG and GLN HCAECs, respectively (P<0.01). Azaserine completely reversed the effects of HG and GLN on both pro-MMP-2 and pro-MMP-9 (P<0.001 for both MMP-9 and MMP-2; Figure 5, a and b). MMP-9 and MMP-2 expression was markedly increased in both HG and GLN HCAECs (P<0.001; Figure 5b). To verify whether increased ERK-1/2 activity observed in HG and GLN HCAECs contributed to enhanced MMP-9 and MMP-2 expression, we incubated cells with PD098059 inhibitor. Inhibition of ERK-1/2 did not significantly reduce the HG- and GLN-dependent increase in activity of MMP-9 and MMP-2 (Figure 5a) and protein levels (data not shown).

**TIMP-3 Levels Are Reduced in HCAECs Incubated With HG and GLN**

TIMPs inhibit activation of pro-MMP to active MMP. Reverse zymography assay of HCAEC culture media revealed no defects in TIMP-1 and TIMP-2 expression (Figure 5c and data not shown). By contrast, TIMP-3 activity was reduced by 88% and 85% in HG and GLN HCAECs, respectively (P<0.001; n=3). TIMP-3 protein expression was reduced by 74% and 84% in HG and GLN HCAECs, respectively (P<0.013; n=3; Figure 5c).

**GlcNAcylation Is Increased in Atherosclerotic Plaque of Diabetic Patients**

We next assessed O-GlcNAcylation of endothelial cells in vivo by immunostaining with RL2 antibody of carotid plaque of representative diabetic patients. O-GlcNAcylation immunoreactivity in small ovarian vessels with RL2 antibody. Increased O-GlcNAcylation immunoreactivity in endothelium, smooth muscle cells, and macrophage/foam cells from representative carotid plaque of diabetic patients. Slight O-GlcNAcylation immunoreactivity in endothelium, smooth muscle cells, and macrophage/foam cells from representative carotid plaque of nondiabetic subjects. No immunoreactivity was detected when primary antibody was omitted (data not shown). Quantification of immunoreactivity signals in cap plaques from diabetic and nondiabetic subjects. Arrows indicate endothelial cell positive for O-GlcNAcylation immunoreactivity. Significant differences among diabetic subjects compared with nondiabetics are indicated (**P<0.0016, t test).
plaque formation from nondiabetic and diabetic subjects. O-GlcNAcylation immunoreactivity was barely detectable in control endothelial cells from nonatherosclerotic small vessels (Figure 6, a through f; Table). The endothelial layer of carotid plaques showed intense signal in both cytoplasm and nuclear compartments of diabetic patients arteries compared with nondiabetic subjects (Figure 6, g and h). No immunoreactivity was detected when the primary antibody was omitted (data not shown). O-GlcNAcylation immunoreactivity in the cap plaque was 6.6-fold increased in diabetic samples ($P<0.0016$; n=4 Figure 6i).

**Discussion**

Activation of the hexosamine pathway has been proposed as a link between glucose toxicity and development of diabetic complications, whereas hyperinsulinemia, secondary to insulin resistance, has been hypothesized to promote the atherogenic effects of insulin. In the present report, we show that activation of the hexosamine biosynthesis in HCAECs determines epistatic defects in the pathway that transduces the insulin effect from the membrane receptor to eNOS activation, by increased O-GlcNAcylation of key signaling molecules. Reduction of eNOS activity is associated with increased expression of MMP-2 and MMP-9 and decreased expression of TIMP-3, which might contribute to the development of macrovascular disease. Increased O-GlcNAcylation of signaling molecules in the PI3-K pathway could also explain increased MAPK activity, because Akt is known to negatively regulate the upstream MAPK activator Raf-1. Thus, O-GlcNAcylation impairment of the metabolic IR/IRS/PI3-K/Akt pathway might impair the protective effects of eNOS and also might promote vascular cell growth and endothelin-1 and plasminogen activator inhibitor-1 overexpression by MAPK hyperactivation.

There is evidence that activation of Akt may exert antiatherogenic effects by phosphorylating eNOS on Ser1177. Prediction of eNOS O-GlcNAcylation sites allowed us to identify several potential sites surrounding Ser1177 but not other phosphorylation sites, such as Thr495 and Ser116 (Figure 3a). We found that treatment of HCAECs with HG and GLN caused a marked reduction in insulin-induced eNOS activity as a result of increased O-GlcNAcylation of key insulin-signaling elements. Interestingly, blockade of the hexosamine pathway activation by azaosine reversed the effect of HG on O-GlcNAcylation levels, insulin-induced activity, and Ser1177 phosphorylation of eNOS. It is conceivable that O-GlcNAcylation of Ser1177 or close residues prevents Akt from phosphorylating and activating eNOS. However, because azaosine is a nonspecific inhibitor of GFAT, the rate-limiting enzyme in hexosamine metabolism, the present data suggest but do not conclusively document causality.

While this article was in preparation, Du et al demonstrated that inhibition of GFAT through antisense diminished HG-dependent O-GlcNAcylation of eNOS. Thus, they provided causal evidence that O-GlcNAcylation of eNOS induced by hyperglycemia impaired basal enzyme activity. Moreover, they observed that mutation of the Akt site Ser1177 resulted in reduction of eNOS O-GlcNAcylation in bovine endothelial cells exposed to high glucose levels. Our data are in agreement with the observation by Du et al and provide new evidence that O-GlcNAcylation could play a role in modulating the dynamics of insulin signaling in the vasculature.

eNOS has been shown to inhibit both MMP-2 and MMP-9 activity through NO production. Interestingly, in muscle and aorta from IR+/- insulin-resistant diabetic mice, we have observed decreased expression and activity of TIMP-3, accompanied by increased MMP-2 and MMP-9 activity (M.F. and D. Accili, MD, unpublished data, 2001). Likewise, in HCAECs, impairment of insulin signaling involving the PI3-K/Akt/eNOS pathway induced by HG or GLN is associated with increased MMP-2 and MMP-9 expression and activity and reduced TIMP-3. By contrast, inhibition of MAPK activity by a selective inhibitor did not modify increased MMP activity observed in this system. To verify whether O-GlcNAcylation is present in the vasculature of diabetic patients, nonatherosclerotic vessels and carotid plaques from both type 2 diabetic and nondiabetic subjects were analyzed. Weak O-GlcNAcylation was observed in endothelium of nonatherosclerotic arteries. By contrast, carotid plaques from diabetic patients revealed a marked increase of O-GlcNAcylation in both cytoplasm and nuclear compartments of endothelial cells compared with nondiabetic subjects. The presence of O-GlcNAcylation in carotid plaques from nondiabetic subjects, although at lower levels, is of interest because it could be speculated to be related to superoxide overproduction by factors other than hyperglycemia. Superoxide production is known to favor hexosamine pathway activation, and there is evidence that inhibition of mitochondrial superoxide generated by HG reduces hexosamine pathway activation and eNOS O-GlcNAcylation.

Glucosamine has been shown recently to suppress inducible NO synthase protein and activity in macrophages activated by inflammatory cytokines. This observation could explain the positive effect of glucosamine in the treatment of arthritis. Although we did not assess the effect of glucosamine on inducible NO synthase in our cell system, there is evidence that glucose toxicity adversely affects diabetic macrovascular complications, and acute infusion of glucosamine in humans reproduces some metabolic features of diabetes.

In conclusion, our study data support the idea that hyperglycemia, acting at least in part through the hexosamine pathway, may contribute to the development of diabetic macrovascular complications by selectively impairing the metabolic branch of insulin signaling that involves the PI3-K/Akt/eNOS pathway.

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