Epidermal Growth Factor Receptor Transactivation Is Regulated by Glucose in Vascular Smooth Muscle Cells*

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We hypothesized that glucose-mediated alterations in vascular smooth muscle cell signal transduction contribute to diabetic complications. We found enhanced AngII activation of Akt and extracellular ERK1/2 in vascular smooth muscle cells incubated with high glucose (27.5 mM) compared with low glucose (5.5 mM). Because AngII-mediated transactivation of the epidermal growth factor receptor (EGFR) is important in Akt and ERK1/2 activation, we studied the effects of glucose on EGFR function. The EGFR in cells cultured for 48 h in low glucose was smaller (145 kDa) than the EGFR in cells cultured with high glucose (170 kDa). The shift from the 170-kDa isoform to the 145-kDa isoform was reversible and dependent upon glucose concentration with EC50 ~1 mM. N-Glycosylation was responsible because peptide N-glycosidase F treatment of isolated 170-kDa EGFR yielded a single band at 145 kDa. Cell surface biotinylation showed that the 145-kDa EGFR was present on plasma membrane. AngII and other G-protein-coupled receptor ligands known to transactivate EGFR phosphorylated the 170-kDa EGFR but not the 145-kDa EGFR, whereas EGF, heparin-binding EGF-like growth factor, and transforming growth factor-α phosphorylated both receptors. Subcellular fractionation showed that the 145-kDa receptor localized to a different plasma membrane domain than the 170-kDa receptor. These results establish a novel mechanism by which glucose-dependent EGFR N-glycosylation modulates AngII signal transduction and suggest a potential mechanism for pathogenic effects of AngII in diabetic vasculopathy.

Vascular smooth muscle cell (VSMC)† function is known to be highly sensitive to glucose concentrations, which may be one reason why diabetes is associated with an increased rate of vascular complications, including atherosclerosis and hypertension (1). A pathogenic interaction between high glucose (HG) and angiotensin II (AngII) seems likely because many clinical studies have demonstrated the effectiveness of inhibiting AngII to limit diabetic complications such as myocardial infarction, stroke, and renal failure (2, 3). The benefits are greater than expected solely from reductions in blood pressure, suggesting a direct AngII mechanism. Indeed, it is reported that HG enhances AngII- or serum-induced VSMC proliferation and prevents VSMC apoptosis (4–6). HG also induces expression of plasminogen activator inhibitor-1 (PAI-1) and further augments PAI-1 expression induced by AngII in VSMC (7, 8). These effects of glucose on VSMC seem to be related to the alterations of intracellular signal transduction because HG enhances mitogen-activated protein kinase (MAPK) activation induced by AngII (9). It has been suggested that MAPK stimulated by HG is secondary to protein kinase C activation by increasing the endogenous activator diacylglycerol (10–12). However, the mechanism remains uncertain, because the increases in diacylglycerol and protein kinase C activation seem too small to explain MAPK activation.

Recently, it has become apparent that AngII signal transduction is mediated in part through epidermal growth factor receptor (EGFR) transactivation (13). Several downstream events of EGFR transactivation including MAPK and Akt activation participate in VSMC proliferation and anti-apoptosis (14, 15). MAPKs also stimulate activator protein-1, a key transcription complex that induces transcription of PAI-1. Indeed, AngII-induced PAI-1 expression is inhibited by AG1478, a tyrosine kinase inhibitor specific for EGFR, indicating EGFR transactivation is essential for PAI-1 expression by AngII (16).

Based on the many signaling events common to EGFR transactivation and HG stimulation, we hypothesized that glucose modulates EGFR transactivation by G-protein-coupled receptors (GPCRs) such as the AngII receptor. We found that glucose dramatically altered EGFR function by post-translational N-glycosylation modifications. Furthermore, we observed that EGFR transactivation by GPCRs (but not EGFR activation by EGF) correlated with glycosylation state and with activation of Akt and MAPK. We propose a novel paradigm in which glucose modulates GPCR and growth factor signal transduction by means of changes in EGFR N-glycosylation.

EXPERIMENTAL PROCEDURES

Materials—Antibodies to phospho-ERK, phospho-Akt (Ser-473), and Akt were purchased from Cell Signaling Technologies (Beverly, MA). Antibodies to AT1R (polyclonal), EGFR (polyclonal), PDGF-BB (polyclonal), and phosphotyrosine (monoclonal) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody to α-actin was obtained from Roche Applied Science, a monoclonal antibody to EGFR from Transduction Laboratories (Lexington, KY), antibody to human EGF-R (Tyr-845) from BioSource International (Camarillo, CA), recombinant human EGF, HB-EGF, and TGF-α from R&D Systems (Minneapolis, MN), sulfo-NHS-biotin and streptavidin-agarose from Pierce, angiotensin II from ICN Biomedicals (Aurora, OH), and all

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‡ The abbreviations used are: VSMC, vascular smooth muscle cell; HG, high glucose; LG, low glucose; AngII, angiotensin II; PAI-1, plasminogen activator inhibitor-1; MAPK, mitogen-activated protein kinase; EGFR, epidermal growth factor receptor; GPCR, G-protein-coupled receptors; ERK, extracellular signal-regulated kinase; TGF, transforming growth factor; DMEM, Dulbecco’s modified Eagle’s medium; PNGase-F, peptide N-glycosidase F; MES, 4-morpholineethanesulfonic acid; AT1R, angiotensin type I receptor; HB-EGF, heparin-binding EGF-like growth factor; PDGF, platelet-derived growth factor.

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other reagents and chemicals were obtained from Sigma, unless specifically indicated.

**Cell Culture—** VSMC were isolated from the thoracic aorta of 200- to 250-g male Sprague-Dawley rats and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen), as described previously (17). VSMC used for the present study were maintained for >3 passages in DMEM containing 5.5 mM glucose.

**Immunoblot Analysis—** Western blot analyses were performed as described previously (18). VSMC were lysed in Triton-based lysis buffer (1% Triton X-100, 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 0.1% protease inhibitor mixture (Sigma)), scraped off the dish, and centrifuged at 10,000 × g for 10 min; the supernatant was collected as a total cell lysate. Equal amounts of cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond™ EDL, Amersham Biosciences). Membranes were incubated with appropriate primary antibodies, and membrane-bound antibodies were visualized by horseradish peroxidase-conjugated secondary antibodies and the ECL system (Amersham Biosciences).

**Immunoprecipitation—** Lysates containing equal amounts of protein were incubated with anti-EGFR antibody (monoclonal) rocking overnight at 4 °C. After incubation with protein G-agarose (Invitrogen) for 2 h, precipitates were washed with lysis buffer and then resuspended in SDS-PAGE sample buffer. After being denatured at 100 °C for 5 min, samples were separated by SDS-PAGE and transferred to nitrocellulose membrane for immunoblotting.

**Glycosidase Digestion—** 300 μg of lysate were immunoprecipitated with anti-EGFR antibody and digested with peptide N-glycosidase F (PNGase-F, New England Biolabs, Beverly, MA). Specifically, samples were incubated in denaturing buffer (0.5% SDS and 1% β-mercaptoethanol) for 10 min at 100 °C and brought to 50 mM sodium phosphate (pH 7.5) with 1% Nonidet P-40. Then, 1 μl (500 units) of PNGase-F was added and incubated 1 h at 37 °C. After glycosidase digestion, SDS-PAGE sample buffer was added and incubated at 100 °C for 5 min.

**Cell Surface Biotinylation—** Cells were washed twice in ice-cold phosphate-buffered saline (150 mM NaCl and 20 mM Na2HPO4, pH 7.4) and once in borate buffer (154 mM NaCl, 10 mM boric acid, 7.2 mM KCl, and 1.8 mM CaCl2, pH 9). The surface plasma membrane proteins were biotinylated by gently shaking the cells for 20 min at 4 °C with borate buffer containing 0.5 mg/ml NHS-SS-biotin. After the same volume of biotinylation solution was added, cells were further rocked for 20 min. Cells were washed extensively with the quenching buffer (20 mM Tris and 120 mM NaCl, pH 7.4) to scavenge the unreacted biotin, then washed twice with phosphate-buffered saline.

**Detergent-free Subcellular Fractionation—** Subcellular fractions were prepared according to a method modified from Song et al. (19). After rinsing with ice-cold phosphate-buffered saline, VSMC were scraped into 2 ml of buffer A (500 mM sodium carbonate, pH 11.0, 25 mM MES, 150 mM NaCl, and 0.2% protease inhibitor mixture) and then homogenized by using a Dounce homogenizer (20 strokes) and a sonicator (3 × 10-s bursts; ultrasonic Homogenizer 4710 series, Cole-Parmer Instrument Co., Chicago, IL). The homogenate was adjusted to 45% sucrose by the addition of 2 ml of buffer B (500 mM sodium carbonate, pH 11.0, 25 mM MES, 150 mM NaCl, and 0.2% protease inhibitor mixture) and then homogenized by using a Dounce homogenizer (20 strokes) and a sonicator (3 × 10-s bursts; ultrasonic Homogenizer 4710 series, Cole-Parmer Instrument Co., Chicago, IL). The homogenate was adjusted to 45% sucrose by the addition of 2 ml of buffer B (90% sucrose, 25 mM MES, pH 6.5, 150 mM NaCl) and placed at the bottom of an ultracentrifuge tube. A discontinuous sucrose gradient was formed by layering 2 ml of 35, 30, 25, 10, and 5% sucrose in buffer C (250 mM sodium carbonate, pH 6.5, 25 mM MES, 150 mM NaCl, and 0.1% protease inhibitor mixture) and centrifuged at 25,000 rpm for 20 h in a Sorvall Discovery™ 100S ultracentrifuge equipped with Surespin™ 650 rotor (Kendro Laboratory Products, Newtown, CT). 12 × 1-ml fractions were collected from
Glucose Modulates Akt and ERK1/2 Phosphorylation upon Stimulation with AngII but Not with EGF—To explore the effect of glucose on AngII signal transduction, we studied Akt and ERK1/2 activation. Akt phosphorylation at Ser-473 was time dependently increased by AngII stimulation with a 3.7 ± 0.6-fold increase at 5 min when cultured in HG (Fig. 1A, left). On the other hand, Akt phosphorylation was completely inhibited in LG (0.54 ± 0.05-fold control at 5 min, p < 0.001 between LG and HG). ERK1/2 phosphorylation was also increased by AngII in HG (Fig. 1A, right) but suppressed in LG (2.7 ± 0.5-versus 5.1 ± 1.0-fold increase at 5 min, p < 0.001). These data indicate that glucose significantly modulates phosphorylation of two independent signaling molecules, Akt and ERK1/2, induced by AngII. In contrast, upon stimulation by EGF, both Akt and ERK1/2 phosphorylation did not differ significantly between LG and HG (Fig. 1B, p > 0.05), suggesting that the glucose effect on signal transduction might be specific to AngII. As shown previously (14, 15), Akt and ERK1/2 phosphorylation upon stimulation with AngII mostly depended on EGFR transactivation, because AG1478 (1 μM), a tyrosine kinase inhibitor specific for EGFR, reduced AngII-induced Akt and ERK1/2 phosphorylation by 111.6% and 78.9%, respectively, in HG-cultured cells (data not shown).

Glucose-dependent Change in EGFR Molecular Mass—Next, we explored upstream targets in the EGFR transactivation pathway to explain the glucose-dependent difference in Akt and ERK1/2 phosphorylation by AngII. Western blotting from VSMC grown in LG and HG showed no difference in angiotensin type I receptor (AT1R) expression (Fig. 2A, middle panel). However, there were two molecular mass isoforms of EGFR (145 and 170 kDa) in LG, whereas only a 170-kDa protein was expressed in HG (Fig. 2A, upper panel). EGFR molecular mass changed in a manner dependent upon incubation time and initial glucose concentration (Fig. 2B). Initially (time = 0), EGFR was present as a 170-kDa protein that is usually reported in VSMC (14). After changing the media to LG (5.5 mM), a 145-kDa EGFR protein became increasingly apparent and finally replaced the 170-kDa form after a 48-h incubation. At an intermediate glucose concentration (11 mM), both receptors were evident, and at HG (27.5 mM), there was no appearance of the 145-kDa form even at 48 h. Because other groups have reported that human VSMC rapidly depletes glucose in culture media (20), we studied glucose consumption by rat VSMC. As shown in Fig. 2C, VSMC consumed more than 50% of the initial glucose within 24 h, suggesting that the change in EGFR molecular mass may occur in response to glucose concentrations lower than 5.5 mM.

Glucose Refeeding after Starvation Induces Reappearance of the 170-kDa Receptor—To further characterize the mechanism of EGFR isoform switching, we grew VSMC without glucose for 24 h and then refeed with various concentrations of glucose. Upon refeeding, there was a rapid reappearance of the 170-kDa EGFR within 6 h (Fig. 3A, upper panel). The reappearance was glucose-dependent, with an EC\textsubscript{50} of ∼1 mM (Fig. 3A, lower panel). We also studied PDGFR under the same conditions and found no change in the molecular mass during the course of starving and refeeding at any glucose concentration, indicating that the glucose effect may be unique to EGFR. To gain insight into the role of glucose metabolism as compared with osmotic stress in the EGFR isoform switch, we compared the abilities of different carbohydrates to induce the 170-kDa EGFR after glucose starvation. As shown in Fig. 3B, metabolically active carbohydrates (α-glucose, mannose, fructose, and galactose)
induced the 170-kDa EGFR form, whereas metabolically inactive sugars (raffinose, mannitol, and L-glucose) did not. This finding suggests that glycolytic processes are required to induce the 170-kDa EGFR.

The Difference between Two Isoforms Is because of Altered N-Glycosylation—The level of N-linked oligosaccharides may change in membrane glycoproteins in response to glucose concentration (21–23). To determine whether the difference between the 145-kDa EGFR and the 170-kDa EGFR was related to N-linked glycosylation, we treated isolated EGFR with PNGase-F to remove carbohydrates from glycopeptides at N-glycosylation sites. As shown in Fig. 4, PNGase-F treatment yielded a single 145-kDa EGFR protein from lysates of LG- and HG-treated cells. This result clearly demonstrates that the core protein is the same between the 145- and the 170-kDa receptor and that the difference is attributed solely to N-linked oligosaccharides.

Cell Surface Localization of the 145-kDa EGFR—An important function of N-linked oligosaccharides is to facilitate proper protein folding. Chemical agents that inhibit N-glycosylation often induce misfolded proteins that are retained in endoplasmic reticulum and never transported to plasma membrane (24). To determine the membrane localization of the 145-kDa EGFR, we labeled the surface of plasma membrane with biotin, then lysed the cells and performed a pull-down assay using streptavidin-agarose. As shown in Fig. 5, both the 145- and the 170-kDa EGFR in LG and HG, respectively, were pulled down from biotinylated samples with ratios comparable with those found.

**FIG. 3. Glucose refeeding induces reappearance of the 170-kDa EGFR.** VSMC were incubated with both serum- and glucose-free DMEM for 24 h. A, cells were refed with the indicated concentration of glucose for 6 h or 24 h. Cell lysates were immunoblotted with anti-EGFR antibody (top panel) or anti-PDGFR antibody (middle panel). The 170-kDa EGFR was quantified by densitometry and is represented in the bottom panel. B, cells were refed with 27.5 mM of D-glucose, mannose, fructose, raffinose, galactose, mannitol, or L-glucose for 6 or 24 h. Cell lysates were immunoblotted with anti-EGFR antibody.
in total cell lysate (Fig. 5, compare lanes 1 and 2 versus lanes 3 and 4). These results demonstrate that the 145-kDa EGFR induced by LG is expressed on the cell surface. Plasma membrane localization of the 145-kDa receptor was further confirmed by EGF-induced phosphorylation and density-dependent fractionation shown below (Figs. 6 and 7).

**The 145-kDa EGFR Can Be Ligand-activated but Not Transactivated**—To gain insight into the function of the 145-kDa EGFR, we explored EGFR tyrosine phosphorylation. Upon stimulation with EGF, both the 170-kDa form and the 145-kDa form were tyrosine-phosphorylated (Fig. 6A, upper panel). When stimulated with AngII, however, there was no 145-kDa receptor phosphorylation, whereas the 170-kDa receptor was still clearly phosphorylated (Fig. 6A, lower panel). To further assess EGFR responsiveness to other ligands, we studied the ability of heparin-binding EGF-like growth factor (HB-EGF) and transforming growth factor α (TGF-α) to stimulate phosphorylation of both isoforms. As shown in Fig. 6B, HB-EGF and TGF-α, like EGF, activated both receptors, suggesting that the 145-kDa EGFR is responsive to ligand-induced activation. To determine whether the inability of the 145-kDa EGFR to be transactivated was a general property, we tested the ability of other GPCR agonists to increase EGFR tyrosine phosphorylation. As shown in Fig. 6C, thrombin and sphingosine 1-phosphate stimulated phosphorylation of the 170-kDa receptor but not of the 145-kDa receptor. These findings suggest that the 145-kDa EGFR is generally unresponsive to transactivation.

To confirm that the difference in AngII-induced tyrosine-phosphorylation of the 145-kDa receptor correlated with EGFR activation, we studied phosphorylation of an activation-specific site in EGFR (25). As shown in Fig. 6D, there was no phosphorylation of Tyr-845 in the 145-kDa receptor upon stimulation with AngII, whereas EGF induced Tyr-845 phosphorylation in both receptors. These results indicate that AngII and other GPCR agonists cannot transactivate the 145-kDa EGFR. In some cell systems, EGFR transactivation depends upon membrane-anchored HB-EGF, which is shed after release by metalloproteases. The HB-EGF then activates EGFR by means of an extracellular pathway (26, 27). Because it is possible that glucose may affect EGFR transactivation through modulating expression and/or shedding of membrane-anchored growth factors, we studied the contribution of the extracellular pathway to EGFR phosphorylation by AngII in our cell system. As shown in Fig. 6E, heparin and CRM197, two molecules that bind and inhibit HB-EGF, could not reduce EGFR phosphorylation at concentrations shown to inhibit HB-EGF function (27), whereas AG1478, a tyrosine kinase inhibitor specific for EGFR, completely inhibited EGFR transactivation by AngII. These results demonstrate that HB-EGF is not a dominant mechanism for EGFR transactivation in our system and thus suggest that the impaired EGFR phosphorylation by AngII in LG is an intrinsic property specific to the 145-kDa EGFR.

**Localization of EGFR within Plasma Membrane**—A previous report demonstrated that disruption of lipid rafts and/or caveolae prevented EGFR transactivation by AngII but not activation by EGF (15). It was also shown that the second cysteine-rich region of EGFR containing two potential N-glycosylation sites was important for targeting EGFR to lipid rafts and/or caveolae (28). Thus, we hypothesized that a change in membrane localization of the 145-kDa EGFR may cause impaired phosphorylation by AngII. To test this possibility, we explored subcellular localization of EGFR by using sucrose density centrifugation. As shown in Fig. 7, there was a significant difference in the distribution of EGFR in LG and HG. Although both receptors were localized within caveolae-enriched fractions collected between 5 and 35% sucrose (29), a much greater percentage of EGFR from HG-treated cells was present in heavier fractions (Fig. 7, A and C, fractions 6–10). In contrast to the change in EGFR location, there were no significant differences in caveolin-1 distribution in LG compared with HG (Fig. 7B). These results suggest that the change in N-glycosylation may specifically alter EGFR location, thereby altering responsiveness to transactivation.

**DISCUSSION**

In the present study, we investigated the modulation of signal transduction by glucose in VSMC. The major findings are that glucose modulates EGFR N-glycosylation; the 170-kDa N-glycosylated EGFR can be phosphorylated in response to both EGF and GPCR ligands (transactivation), whereas the 145-kDa EGFR can only be phosphorylated in response to EGF; glucose-modulated EGFR transactivation is required for activation of downstream signaling events such as Akt and ERK1/2. To our knowledge, this is the first report to show that altered N-glycosylation modulates EGFR transactivation.

Recent studies show that many GPCRs, including the AT1R, transactivate growth factor receptors, including the EGFR and the PDGF receptor (30, 31). Transactivation is important for many of the VSMC effects reported for AngII, including proliferation, hypertrophy, and migration (32–34). EGFR transactivation is a Ca++/calmodulin-dependent process that involves the endogenous EGFR tyrosine kinase, Pyk2, and reactive oxygen species (30). The present study shows that another requirement in VSMC is the presence of an N-glycosylated 170-kDa EGFR that depends upon glucose concentration.

Previous publications have shown that glucose modulates various signaling events in VSMC, including activation of ERK1/2 and p38 (9). HG has also been reported to enhance VSMC proliferation when stimulated with AngII by unknown mechanisms (4). In fact, other GPCR-coupled ligands, such as sphingosine 1-phosphate, thrombin, and lysophosphatidic acid,
**Fig. 6.** EGFR phosphorylation upon stimulation with various agonists. VSMC were incubated with serum-free DMEM containing either LG (5.5 mM) or HG (27.5 mM) for 48 h. **A**–**C** and **E**, after stimulation, cells were lysed, and the lysates were immunoprecipitated with anti-EGFR antibody (monoclonal) and immunoblotted with anti-phosphotyrosine antibody (*top*); cells were then reprobed with anti-EGFR antibody (polyclonal) (*bottom*). **A**, VSMC were stimulated with EGF (20 ng/ml, *upper*) or AngII (100 nM, *lower*) for indicated times. **B**, VSMC were stimulated with HB-EGF (20 ng/ml), TGF-α (20 ng/ml), or EGF (20 ng/ml) for 2 min. **C**, cells were stimulated with thrombin (1 unit/ml, 2 min), sphingosine-1-phosphate (SIP) (1 μM, 1 min). **D**, VSMC were stimulated with AngII (100 nM, 1 min) or EGF (20 ng/ml, 2 min), and cell lysates were blotted with anti-phospho-EGFR (Y845) specific antibody; cells were then reprobed with anti-EGFR antibody (polyclonal). **E**, VSMC serum-starved in HG were incubated with CRM197 (10 μg/ml), heparin (100 μg/ml), or AG1478 (1 μM) for 30 min and then stimulated with AngII (100 nM, 1 min).

**Fig. 7.** EGFR distribution defined by sucrose density centrifugation. VSMC were incubated with serum-free DMEM containing either LG (5.5 mM) or HG (27.5 mM) for 48 h. VSMC were collected and fractioned as described in “Materials and Methods.” Cell lysates were immunoblotted with anti-EGFR antibody (**A**) or anti-caveolin-1 antibody (**B**). **C**, densitometry data were quantified and expressed as a % of total EGFR in each fraction. Values are mean ± S.E. for three independent experiments.
Glucose Regulation of EGFR Transactivation

Fig. 8. Model for effects of glucose on EGFR and AngII-mediated transactivation.

...share with AngII the following properties: transactivation of EGFR, activation of ERK1/2 and Akt, and promotion of VSMC proliferation and survival (13–15). Based on the present study, we propose a critical role for glucose modulation of EGFR transactivation by GPCR agonists in VSMC (Fig. 8). This mechanism is unique to transactivation, because we found that EGFR ligands, such as EGF, HB-EGF, and TGF-α, were able to activate both forms of EGFR.

A previous study reported altered glycosylation of EGFR in a human carcinoma cell line after glucose starvation induced by 2-deoxyglucose (35). Although the molecular mass (150 kDa) of EGFR in the report by Cai et al. (35) was similar to the present paper, the 145-kDa EGFR in VSMC grown in LG is physiologically quite different from the chemically induced receptor for two reasons. First, the 145-kDa EGFR in LG-cultured VSMC is expressed on the cell surface (Fig. 5, 7), whereas the receptor in 2-deoxyglucose-treated cells is not. Second, the LG-induced EGFR is fully functional upon stimulation with classical ligands (Figs. 6, A and B) and able to transduce downstream signals (Fig. 1B), whereas the 2-deoxyglucose-induced receptor has no function. From these results, we conclude that the 145-kDa EGFR that appears in LG is physiologically “allowed” to insert in the plasma membrane. We speculate that it has an alternative function such as impaired response to GPCR-induced transactivation (Fig. 6, A and C). In fact, among several membrane proteins known to be modulated on the N-linked carbohydrates in a glucose-dependent manner, most are not expressed on the cell surface and subsequently are not functional in low glucose conditions (21, 22). The only other receptor known to locate on the cell surface in the presence of both LG and HG and that shows different functions depending on glucose is CD44 (23). The glucose level required to change CD44-N-glycosylation in Chinese hamster ovary-K1 cells is ~1.4 mM, which is comparable with our results (Fig. 3A). These data suggest that there might be common mechanisms for functional regulation of EGFR and CD44 based on glucose-mediated alterations in N-glycosylation.

The mechanism by which altered N-glycosylation modulates EGFR transactivation remains to be clarified. We believe that a likely mechanism is a change in membrane microdomain location that affects signal transduction. There are 10 potential N-glycosylation sites in full-length rodent EGFR, and all N-glycan sites are conserved in the human receptor, emphasizing the importance of glycosylation to EGFR function. Although N-linked oligosaccharides may both positively and negatively regulate EGFR function (based on published data; see Refs. 36 and 37), the direct link between N-glycosylation and plasma membrane domain localization remains to be elucidated. Some investigators have shown that depletion of plasma membrane cholesterol impairs EGFR transactivation, suggesting that transactivation requires targeting EGFR to caveolae and/or lipid rafts (15). The present result, in which there was a significant difference in EGFR distribution within caveolin-enriched fractions between LG- and HG-cultured cells (Fig. 7), suggests that there might be a requirement of N-linked oligosaccharides for the targeting of EGFR to a specific microdomain in which the receptor can be transactivated.

The novel finding that glucose modulates EGFR transactivation through altered N-glycosylation has significant implications for our understanding of basic mechanisms of VSMC signal transduction and clinical consequences of hyperglycemia. Because EGFR is involved in many of the intracellular signaling pathways activated by GPCRs, alterations in EGFR N-glycosylation will have significant effects on GPCR function. Moreover, because EGFR has no function. From these results, we conclude that the 145-kDa EGFR that appears in LG is physiologically “allowed” to insert in the plasma membrane. We speculate that it has an alternative function such as impaired response to GPCR-induced transactivation (Fig. 6, A and C). In fact, among several membrane proteins known to be modulated on the N-linked carbohydrates in a glucose-dependent manner, most are not expressed on the cell surface and subsequently are not functional in low glucose conditions (21, 22). The only other receptor known to locate on the cell surface in the presence of both LG and HG and that shows different functions depending on glucose is CD44 (23). The glucose level required to change CD44-N-glycosylation in Chinese hamster ovary-K1 cells is ~1.4 mM, which is comparable with our results (Fig. 3A). These data suggest that there might be common mechanisms for functional regulation of EGFR and CD44 based on glucose-mediated alterations in N-glycosylation.

The mechanism by which altered N-glycosylation modulates EGFR transactivation remains to be clarified. We believe that a likely mechanism is a change in membrane microdomain location that affects signal transduction. There are 10 potential N-glycosylation sites in full-length rodent EGFR, and all N-glycan sites are conserved in the human receptor, emphasizing the importance of glycosylation to EGFR function. Although N-linked oligosaccharides may both positively and negatively regulate EGFR function (based on published data; see Refs. 36 and 37), the direct link between N-glycosylation and plasma membrane domain localization remains to be elucidated. Some investigators have shown that depletion of plasma membrane cholesterol impairs EGFR transactivation, suggesting that transactivation requires targeting EGFR to caveolae and/or lipid rafts (15). The present result, in which there was a significant difference in EGFR distribution within caveolin-enriched fractions between LG- and HG-cultured cells (Fig. 7), suggests that there might be a requirement of N-linked oligosaccharides for the targeting of EGFR to a specific microdomain in which the receptor can be transactivated.

The novel finding that glucose modulates EGFR transactivation through altered N-glycosylation has significant implications for our understanding of basic mechanisms of VSMC signal transduction and clinical consequences of hyperglycemia. Because EGFR is involved in many of the intracellular signaling pathways activated by GPCRs, alterations in EGFR N-glycosylation will have significant effects on GPCR function. Clinically, it has become apparent that inhibition of AngII by angiotensin-converting enzyme inhibitors or AngII receptor blockers has a dramatic benefit in reducing cardiovascular events and renal failure in patients with diabetes, suggesting an important interaction between AngII and hyperglycemia (2, 3). Based on the present study, we may speculate that inhibitors of EGFR N-glycosylation may have important benefits in cardiovascular disease by limiting GPCR-mediated growth factor receptor transactivation.

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