De novo emergence of insulin-stimulated glucose uptake in human aortic endothelial cells incubated with high glucose

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Gosmanov, Aidar R., Frankie B. Stentz, and Abbas E. Kitabchi. De novo emergence of insulin-stimulated glucose uptake in human aortic endothelial cells incubated with high glucose. Am J Physiol Endocrinol Metab 290: E516–E522, 2006. First published October 25, 2005; doi:10.1152/ajpendo.00326.2005.—Elevated glucose concentrations have profound effects on cell function. We hypothesized that incubation of human aortic endothelial cells (HAEC) with high glucose increases insulin signaling and develops the appearance of insulin-stimulated glucose uptake by the cells. Compared with 5 mM glucose, incubation of HAEC with 30 mM glucose for up to 48 h increased in a time-dependent manner expression of insulin receptor, insulin receptor substrate (IRS)-1, IRS-2, and GLUT1 proteins. High glucose also increased the specific binding of 125I-labeled insulin in HAEC accompanied by accelerated production of interleukin (IL)-6 and IL-8. Short-term stimulation by 50 U/ml insulin did not activate 14C-glucose uptake by HAEC incubated in 5 mM glucose. However, an addition of insulin to high glucose-exposed endothelial cells led to a significant increase in [14C]glucose uptake in a glucose concentration- and time-dependent fashion, reaching a plateau at 48 h of incubation. Furthermore, incubation of HAEC with 30 mM glucose resulted in a new insulin-stimulated extracellular signal-regulated kinase-1/2 mitogen-activated protein kinase phosphorilation and increased lipid peroxidation and production of reactive oxygen species. These studies show for the first time that high glucose increases expression of insulin receptors and downstream elements of the insulin-signaling pathway and transforms “insulin-resistant” aortic endothelial cells into “insulin-sensitive” tissue regarding glucose uptake.

insulin resistance; hyperglycemia; diabetes mellitus; insulin receptor; activated endothelial cells

IT IS CLEAR THAT STRICT GLUCOSE CONTROL results in reduction in risk of microvascular complications in diabetes mellitus (44). The relationship between hyperglycemia and macrovascular complications, however, is less obvious despite the fact that subjects with type 2 diabetes are two to four times more prone to develop cardiovascular pathology than nondiabetic subjects (15, 41). Hyperglycemia is recognized as a crucial factor contributing to vascular impairment in diabetes mellitus, obesity, and metabolic syndrome (9, 14, 44, 45a, 46). A large body of evidence indicates that in hyperglycemic conditions one of the major contributors to the development of large vessel pathology is the endothelial dysfunction (9, 10, 16).

It has been shown that high glucose impairs vasodilatory action of insulin by decreasing the ability of endothelial cells to activate the endothelial nitric oxide synthase-nitric oxide pathway (11, 33). Indeed, previous in vivo and in vitro studies demonstrated that hyperglycemia impairs insulin-induced vasorelaxation (34, 43, 47). The mechanism(s) by which high glucose promotes endothelial dysfunction is via excessive intracellular glucose accumulation and oxidative stress, which lead to increased production of reactive oxygen species (ROS) with consequent induction of the aldose reductase pathway and formation of advanced glycation end products (AGEs) as well as enhanced signaling at the level of protein kinase C (PKC) and mitogen-activated protein kinases (MAPK; see Refs. 9, 31, 35).

Aortic endothelial cells do express insulin receptors (InsR) with characteristics similar to those in other tissues, and GLUT1 is the major glucose-transporting protein in endothelial cells (3, 4, 8, 18, 19, 28). However, insulin does not increase glucose transport by aortic endothelial cells incubated in medium containing 5 mM glucose (3, 8, 20), which might be explained by either a relatively low concentration of InsR in the cell membrane or by an inability of physiological doses of insulin to initiate a signaling cascade (1, 8, 20).

One of the well-established effects of oxygen radicals is an acceleration of cellular growth (6). For instance, vascular endothelial and smooth muscle cell genotypes are dramatically reversed to proatherosclerotic by oxidative stress, which is accompanied by secretion of vascular growth factors (5). Also, it was demonstrated that oxidative stress in aortic endothelial cells promotes synthesis and secretion of proinflammatory cytokines interleukin-6 (IL-6) and interleukin-8 (IL-8), which were implicated in pathogenesis of atherosclerosis (36, 37, 42). Furthermore, studies by our laboratory also demonstrated that prooxidative action of hyperglycemia leads to emergence of InsR and insulin-signaling intermediates in T lymphocytes both in vitro and in vivo (22, 39, 40), providing background for similar studies utilizing blood vessel cells.

Despite extensive research concerning consequences of hyperglycemia on vascular endothelium (12, 31), we lack knowledge whether, similar to T lymphocytes (22), high glucose could promote endothelial responsiveness to insulin. Hence, we hypothesized that exposure of endothelial cells to high glucose upregulates the insulin-signaling pathway, which, in turn, promotes insulin action in regard to glucose uptake.

MATERIALS AND METHODS

Cell culture. Human aortic endothelial cells (HAEC) were purchased from Cambrex (Walkersville, MD) and grown in endothelial cell growth medium-2 BulletKit according to the manufacturer’s recommendations. The cells were seeded at the bottom surface of 25-cm² tissue culture flasks and grown at 37°C in 95% air-5% CO₂ to confluence. Culture medium was changed every 24 h. Endothelial
cells between third and sixth passages were taken for experiments. The cells were incubated either in regular medium containing 5 mM glucose, in medium with high glucose concentration (15 or 30 mM glucose), or in regular medium supplemented with 25 mM mannitol for osmotic control for 6, 12, 24, 48, or 72 h. For each experiment, cells incubated in 5 mM glucose were grown to be used as control and followed over the same time as experimental cells.

Glucose transport. For measurements of 2-deoxy-d-[14C]glucose uptake, experimental media were replaced, and the cells were incubated for 30 min at 37°C in the absence or presence of human insulin as a concentration of 50 μU/ml (0.3 nM). Next, HAEC were incubated for an additional 10 min with 1 μCi/ml 2-deoxy-d-[14C]glucose (New England Nuclear, Boston MA), and uptake was stopped by washing the cells three times with ice-cold PBS. Cells were lysed, scraped, and collected for determination of protein concentration and radioactivity counting. Data were expressed as a degree of change compared with control cells incubated in medium with 5 mM glucose in the absence of insulin.

125I-labeled insulin binding. Confluent HAEC were incubated with 125I-labeled insulin (PerkinElmer Life Sciences, Boston, MA) for 4 h at 30°C. Unlabeled insulin was added in concentrations of 0.01, 0.1, 1, 10, 100, and 1,000 ng/ml. Next, the cells were washed with PBS and solubilized with 0.1% SDS. The radioactivity was measured in a gamma counter and normalized to protein concentration in the samples.

Immunoblotting. HAEC were incubated for 12, 24, or 48 h in medium containing either 5 or 30 mM glucose. Whole cell lysate was prepared by removing experimental medium and washing cells three times with ice-cold PBS followed by incubation for 30 min in ice-cold lysis buffer containing 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 50 mM NaCl, 30 mM Na2HPO4, 50 mM NaF, 100 μM Na3VO4, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml apotinin, 2 μg/ml leupeptin, 2 μg/ml antipain, and 1 μg/ml pepstatin A and centrifuged at 4°C for 15 min at 30,000 g. Protein concentration of the supernatant was measured by the bicinchoninic acid assay (Pierce, Rockford, IL).

Equal amounts of protein were mixed with SDS denaturing buffer, warmed to 95°C for 5 min, electrophoresed on a 10% SDS-PAGE gel, and electroblotted on polyvinylidene difluoride membranes. The membranes were incubated overnight at 4°C in blocking buffer (1.5 mM Na2HPO4, 8 mM NaH2PO4, 0.15 M NaCl, and 0.3% Triton X-100, pH 7.4) supplemented with 3% BSA. Next, the membranes were incubated at room temperature for 1.5 h in blocking buffer containing 1% BSA and the specific antibody. After incubation with 1% BSA blocking buffer containing horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG, the proteins of interest were visualized by chemiluminescent exposure of X-ray film (ECL Plus).

Phosphospecific antibodies to extracellular signal-regulated kinase (ERK)1/2 MAPK dually phosphorylated on Thr202 and Tyr204 (Cell Signaling Technology, Beverly, MA) were used to detect the catalytically activated form of the kinases. Total ERK1/2 MAPK expression was determined by stripping and reprobing the Western blots with antibodies against the anti-total-ERK1/2 MAPK antibody. To measure abundance of the InsR, insulin receptor substrate-1 (IRS-1), IRS-2, GLUT1, p38 MAPK, and protein kinase B (Akt), equal amounts of protein were electrophoresed, and polyvinylidene difluoride membranes were probed with anti-InsR, anti-IRS1, anti-IRS2, anti-GLUT1 antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA), anti-p38 MAPK, or anti-total-Akt antibodies (Cell Signaling Technology). Bands were quantitated by video densitometry. ERK1/2 MAPK phosphorylation was calculated as the ratio of phospho-to-total protein expression and normalized to the control level of expression at 5 mM glucose (taken as 1.0).

Detection of markers of oxidative stress, lipid peroxidation, and cytokines. Assays for markers of oxidative stress and lipid peroxidation in media collected after incubation of HAEC with either 5 or 30 mM glucose for 72 h in the presence or absence of 30-min exposure to insulin were determined by thiobarbituric acid assay and reported as malondialdehyde (21) and by dichlorofluorescein (DCF) assay (39). For further analysis of certain cytokine concentrations, HAEC incubation media collected before the assessment of glucose uptake by the cells was kept at −80°C. Levels of tumor necrosis factor-α (TNF-α), IL-6, and IL-8 were measured in the experimental media using a solid-phase, two-site sequential chemiluminescent immunometric assay on an Immulite analyzer (Diagnostic Products, Los Angeles, CA). The coefficients of variation of the assays were all <5%. The instrument calibrations for the assays were performed as recommended by the manufacturers and were within the specifications.

Statistics. All experiments were done in duplicate at least three times. Comparisons within and among treatments were made by ANOVA and analysis of covariance. Differences between treatments were considered significant at P < 0.05. Data are reported as means ± SE.

RESULTS

Expression of insulin-signaling molecules and GLUT1 in HAEC incubated in high glucose. Incubation of HAEC with 30 mM glucose for 12, 24, and 48 h significantly increased expression of InsR by 13, 35, and 47%, respectively, compared with the values obtained in the cells incubated in 5 mM glucose (Fig. 1A). Similarly, high glucose action resulted in significant elevation of IRS-1 expression of 1.6- to 2.8-fold and IRS-2 expression of 1.6- to 2.3-fold after 12, 24, and 48 h of incubation (Fig. 1, B and C). We also analyzed expression of GLUT1, which is known as a major protein promoting glucose transport in endothelial cells (28). High glucose concentration induced a significant increase in GLUT1 expression of 1.9- to 2.7-fold after 12, 24, and 48 h of incubation (Fig. 1D). We also analyzed expression of other insulin-dependent signal transduction proteins. High glucose did not significantly alter expression of Akt, p38 MAPK, and ERK1/2 MAPK (Fig. 2).

125I-insulin binding in HAEC incubated in high glucose. The abundance of InsR in HAEC was also determined by binding studies. The specific binding of 125I-insulin was very low in control endothelial cells. When HAEC were incubated in 30 mM glucose for 48 h, a significant elevation of insulin-binding capacity of the cells was seen, which could be displaced by increasing concentrations of nonlabeled insulin (Fig. 3).

Production of cytokines by HAEC in high glucose. We found that high glucose did not change production of TNF-α by endothelial cells (5 mM glucose 23.8 ± 1.0 pg/ml; 30 mM glucose at 12 h 21.1 ± 1.3 pg/ml, 24 h 24.4 ± 0.5 pg/ml, and 48 h 24.0 ± 2.75 pg/ml). It is of note that prolonged incubation of HAEC with 30 mM glucose resulted in significantly increased secretion of IL-6 and IL-8. The concentrations of IL-6 increased 1.2- to 2.2-fold in a time-dependent manner (Fig. 4A). In contrast, IL-8 presence in the media was first significantly upregulated after 24 h of high glucose exposure by 2.1-fold and was not changed upon further incubations (Fig. 4B). Incubation of HAEC in medium containing 25 mM mannitol for osmotic control did not produce any changes in cytokine production by the endothelial cells.

Insulin-mediated glucose uptake by HAEC incubated in high glucose. The dramatic increases in intracellular elements are responsible for insulin action that warranted further studies to determine the insulin effect on glucose transport in HAEC. Insulin was not able to stimulate glucose transport in HAEC incubated in 5 mM glucose (Fig. 5A). Basal glucose uptake in endothelial cells incubated in media supplemented with either 15 or 30 mM glucose for 24 h was the same as with 5 mM glucose.
glucose-containing medium. However, when HAEC exposed to high glucose concentrations were incubated with 50 \mu U/ml insulin, we found significant 4.2- and 14.3-fold increases in glucose transport compared with basal values after exposure to 15 and 30 mM glucose, respectively (Fig. 5A).

We further investigated the time course of 30 mM glucose effects on insulin action in HAEC. Insulin stimulation of glucose uptake was not observed after 6 h of incubation with 30 mM glucose (Fig. 5B). However, after 12 h of high glucose exposure, there was a gradual increase in insulin-mediated glucose transport, which reached a plateau after 48 h of incubation. Short-term insulin action in these conditions led to 6.5-, 16.7-, 20-, and 20.8-fold increases in glucose transport after 12, 24, 48, and 72 h, respectively (Fig. 5B).

To rule out the possibility of osmotic effects of high glucose, we supplemented medium containing 5 mM glucose with 25 mM mannitol and incubated HAEC in such a hyperosmolar medium for 24, 48, and 72 h. Neither basal nor insulin-stimulated glucose uptake was changed when compared with control HAEC exposed to mere 5 mM glucose (Fig. 5C).

**Insulin effects on ERK1/2 MAPK phosphorylation, lipid peroxidation, and ROS formation in HAEC**

Acute alterations of an insulin effect on glucose uptake by high glucose in the absence of such changes without insulin suggested that insulin may elicit intracellular effects that are dynamic and appear only upon insulin action. Activations of Akt and p38 MAPK can be observed up to 6.5 h of high glucose incubation. However, ERK1/2 MAPK activation was not observed until 48 h of high glucose exposure. This suggests that high glucose-induced insulin resistance may involve the activation of ERK1/2 MAPK.

**Fig. 1.** Time course of high glucose effect on insulin receptor (InsR; A), insulin receptor substrate (IRS)-1 (B), IRS-2 (C), and GLUT1 (D) expression in human aortic endothelial cells (HAEC). After incubation in 5 or 30 mM glucose, the equal amounts of protein from whole cell lysate were subjected to Western blot analysis with an appropriate antibody. Representative blots are shown. The intensity of each band was quantified and normalized to the level of expression at 5 mM glucose (taken as 1.0). Data are means ± SE. *P < 0.05 compared with expression of InsR at 5 mM glucose.

**Fig. 2.** Time course of high glucose effect on protein kinase B (Akt), p38 mitogen-activated protein kinase (MAPK), and extracellular signal-regulated kinase (ERK)1/2 MAPK expression in HAEC. After incubation in 5 or 30 mM glucose, the equal amounts of protein from whole cell lysate were subjected to Western blot analysis with appropriate antibody. Representative blots are shown.

**Fig. 3.** Effect of high glucose on specific 125I-labeled insulin binding in HAEC. Endothelial cells were incubated with either 5 or 30 mM glucose for 48 h. Next, cells were incubated with 125I-insulin and increasing concentrations of unlabeled insulin. Results are given as specific bound radioactivity to total (100%) radioactivity added.
tion of ERK1/2 MAPK cascade is thought to be one of the mechanisms by which glucotoxicity mediates its deleterious effects (35). Interestingly, high glucose by itself did not alter phosphorylation of ERK1/2 MAPK in HAEC exposed to 30 mM glucose. However, stimulation of the endothelial cells with insulin led to a significant activation of ERK1/2 MAPK phosphorylation of 1.7-, 2.2-, and 2.9-fold after 12, 24, and 48 h of incubation with 30 mM glucose, respectively (Fig. 6).

High glucose increased the intensity of lipid peroxidation and oxidative stress in HAEC; however, insulin did not change either DCF intensity or formation of malondialdehyde (Fig. 7).

DISCUSSION

This is the first report that demonstrates that, in HAEC, high glucose can also increase expression of insulin-signaling proteins accompanied by de novo development of insulin-mediated glucose uptake.

High glucose incubations caused significant stimulation in expression of early elements of the insulin-signaling pathway and GLUT1 (Fig. 1). Naive HAEC exposed to 5 mM glucose, although expressing InsR, do not exhibit an increase in glucose transport in response to either physiological or pharmacological concentrations of insulin (see Fig. 5 and Refs. 8 and 20). However, primed with high glucose concentrations, the endothelial cells displayed a significant increase in insulin-stimulated glucose uptake in relation to glucose concentration and in a time-dependent manner (Fig. 5). This observation was in addition to the extensive studies by Giardino et al. (12) and Nishikawa et al. (31), which showed ROS production by high glucose in aortic endothelial cells in vitro, which was also confirmed in our experimental settings, and work of Mohanty et al. (30), which demonstrated formation of ROS by leukocytes after glucose challenge in vivo. Our results extend these findings in HAEC by showing a combination of high glucose and insulin-stimulated glucose uptake, which did not occur

Fig. 4. Time course of high glucose effect and osmolarity on interleukin (IL)-6 (A) and IL-8 (B) production in HAEC. After incubation in 5 or 30 mM glucose or 25 mM mannitol, the media were collected, and secreted cytokines concentrations were measured by chemiluminescent immunometric assay. Data are means ± SE. *P < 0.05 compared with expression at 5 mM glucose.

Fig. 5. Effect of increasing glucose concentrations on basal and insulin-stimulated glucose transport in HAEC. Endothelial cells were incubated in medium containing either 5, 15, or 30 mM glucose for 24 h (A), in the medium containing 30 mM glucose (B), or in regular medium containing 25 mM mannitol (C). Thereafter, the cells were incubated in the presence or absence of insulin. Glucose uptake was determined as 2-deoxy-d-[14C]glucose uptake transport. Data are means ± SE. *P < 0.001 compared with own basal glucose uptake.
with hyperglycemia alone, thus demonstrating a hitherto new property of endothelial cells.

Aortic endothelial cells express both InsR and insulin-like growth factor I (IGF I) receptor in a 1:10 ratio (3, 4, 25) with the binding affinity of insulin to IGF I receptor ~100 times less than for InsR (48). Interestingly, in contrast to insulin, promitogen IGF I increases glucose transport in HAEC that were not exposed to high glucose (8). One would consider that it may well be IGF I receptor activation that mediates glucose transport by insulin in our experiments. However, a twofold magnitude of increase of glucose transport by maximal concentrations of IGF I in HAEC (3, 8), compared with the significant effects of physiological concentrations of insulin in the presence of high glucose (Fig. 5), argues against involvement of IGF I receptor activation in our settings. Rather, the molecular mechanism that could underlie such a transforming action of high glucose is suggestive of time-dependent overexpression of proximal elements of the insulin-signaling pathway and GLUT1 (Fig. 1) and increased insulin binding to the endothelial cell membrane (Fig. 3). However, the presence of high glucose was not sufficient to elicit an upregulation of glucose uptake because, in basal conditions, HAEC exposed to high glucose did not show any increase in glucose transport (Fig. 5), a finding consistent with previous reports by others (2, 19). This phenomenon can be explained by the fact that high glucose induces the number of GLUT1 transporters that are dormant and become active only upon insulin exposure. Future studies are needed to find whether high glucose concentrations could stimulate GLUT4 protein expression in HAEC, although current evidence indicates the absence of GLUT4 expression in normoglycemic conditions (19, 28, 32).

Recent studies indicate that in vivo hyperglycemia sensitizes endothelial ERK1/2 MAPK to action of several hormones, including insulin (23). Addition of insulin to the HAEC led to a significant increase in ERK1/2 MAPK phosphorylation in a pattern similar to that in glucose transport studies and insulin-signaling molecule expression (Figs. 5 and 6). In contrast, insulin did not affect the accelerated pace of lipid peroxidation and ROS formation by HAEC in high-glucose medium (Fig. 7), ruling out acute in vitro effects of insulin on oxidative stress in hyperglycemic conditions.

Hyperglycemia-induced pathways of endothelial damage can activate the ERK1/2 MAPK cascade either via PKC or AGE production (35). ERK1/2 MAPK is a unique signaling system responsible for cellular growth. In high glucose-activated HAEC, insulin was able to stimulate ERK1/2 MAPK phosphorylation, suggesting that transient glucose exposure sensitizes these cells to insulin’s mitogenic action (Fig. 6). Extensive research by others indicates that insulin action is potentiated in the presence of ROS (27). It has been suggested that ROS-induced inhibition of protein tyrosine phosphatases and/or phosphatases of mitogen-MAPK may “prolong” the life of activated insulin-dependent signaling proteins, thereby enhancing insulin action (13, 27). Nevertheless, further studies are warranted on the detailed molecular mechanism by which high glucose sensitizes HAEC to insulin action.

Indeed, hyperglycemia may act on mammalian cells as a growth factor, and it is possible that production of superoxide may play a significant role (6). Mohanty et al. (30) have previously presented strong evidence for association of ROS production by leukocytes and transient hyperglycemia in vivo. The work by our laboratory clearly shows that, in vitro and in vivo, high glucose promotes expression of receptors to insulin and its downstream signaling elements in T lymphocytes (22, 38, 39). In human T lymphocytes, high glucose exposure increases expression of InsR, IRS-1, GLUT4, proinflammatory cytokines, and oxidative stress components (38). We and others (22, 26, 40) have also demonstrated stimulation of the T lymphocytes and endothelial cell genome by hyperglycemia, which was mediated by ROS, PKC, and MAPK pathways. In addition, hyperglycemia markedly increases the expression of InsR, IRS-1, and IRS-2 in retina and liver of diabetic mice (23).
Elaboration of certain cytokines by aortic endothelial cells in hyperglycemic conditions may be involved in the pathogenesis of endothelial dysfunction in diabetes mellitus (1, 42). In accord with previous studies (36, 37, 42), our findings showed that hyperglycemia in endothelial cells results in elevated production of cytokines (Fig. 4). Pekala et al. (32) showed that incubation of bovine aortic endothelial cells with TNF-α results in a threefold increase in hoxese transport also accompanied by an increased amount of GLUT1 mRNA. Hence, it is possible that cytokines may also contribute to the appearance of insulin-mediated glucose uptake found in our experiments.

On the basis of present findings, it appears that HAEC exhibit a defense mechanism against hyperglycemic damage by providing “gates” to control the inflow of glucose with as little as 15 mM glucose. This along with the intracellular defense systems such as vitamin E, vitamin C, glutathione peroxidase, and superoxide dismutase may thereby be able to fend off deleterious effects of hyperglycemia. However, in the face of other risk factors such as hyperlipidemia and inflammation present in the diabetic state, the defense system may become overwhelmed, resulting in undesirable effects of chronic hyperglycemia (29, 45). In conditions of acute hyperglycemia, there is a marked increase in production of ROS and elevation of levels of cardiovascular markers and proinflammatory markers in the patient’s blood, skeletal muscle, and liver that subside with insulin therapy and euglycemia (22, 24, 39). Furthermore, insulin infusion in patients with acute myocardial infarction demonstrates similar anti-inflammatory effect (7).

We therefore provide experimental evidence that both HAEC and T lymphocytes in response to high glucose increase expression of InsR concomitant with production of ROS and lipid peroxidation, thereby generating insulin responsiveness to ensure whole body glucose homeostasis. However, studies on endothelial cells indicate that such an increased glucose flux could have hazardous outcomes triggering diabetic vascular complications (31).

In conclusion, we have shown for the first time that transient incubation of HAEC with high glucose results in de novo emergence of insulin-mediated glucose uptake in a glucose concentration- and time-dependent manner. The high-glucose effect is associated with a concomitant increase in expression of InsR and molecules responsible for downstream intracellular insulin action. These data demonstrate a possible link between diabetic cardiovascular complications and an appearance of novel insulin effects in aortic endothelium triggered by hyperglycemia. In particular, high glucose concentration-dependent effects could have clinical importance in conditions of critically high blood glucose levels as well as in diabetic patients with suboptimal glycemic control.

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REFERENCES