Estradiol attenuates high glucose-induced endothelial nitrotyrosine: role for neuronal nitric oxide synthase

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Hyperglycemia in diabetes causes increased oxidative stress in the vascular endothelium with generation of free radicals such as superoxide. Peroxynitrite, a highly reactive species generated from superoxide and nitric oxide (NO), induces proinflammatory tyrosine nitration of intracellular proteins under such conditions. The female sex hormone estrogen appears to exert protective effects on the nondiabetic endothelium. However, several studies show reduced vascular protection in women with diabetes, suggesting alterations in estrogen signaling under high glucose. In this study, we examined the endothelial effects of estrogen under increasing glucose levels, focusing on nitrotyrosine and peroxynitrite. Human umbilical vein endothelial cells were incubated with normal (5.5 mM) or high (15.5 or 30.5 mM) glucose before addition of estradiol (E2, 1 or 10 nM). Selective NO synthase (NOS) inhibitors were used to determine the role of specific NOS isoforms. Addition of E2 significantly reduced high glucose-induced increase in peroxynitrite and consequently, nitrotyrosine. The superoxide levels were unchanged, suggesting effects on NO generation. Inhibition of neuronal NOS (nNOS) reduced high glucose-induced nitrotyrosine, demonstrating a critical role for this enzyme. E2 increased nNOS activity under normal glucose while decreasing it under high glucose as determined by its phosphorylation status. These data show that nNOS contributes to endothelial peroxynitrite and subsequent nitrotyrosine generation under high glucose, which can be attenuated by E2 through nNOS inhibition. The altered regulation of nNOS by E2 under high glucose is a potential therapeutic target in women with diabetes.

DIABETES IS A MAJOR RISK FACTOR for cardiovascular diseases (8, 23). Inflammatory changes in the vascular endothelium lead to atherosclerosis that underlies such disease conditions. Hyperglycemia associated with diabetes generates increased oxidative stress in the vascular endothelium. In endothelial cells, hyperglycemia can produce increased levels of the reactive oxygen species (ROS) such as superoxide (O2−), which reacts with endogenous nitric oxide (NO) to yield peroxynitrite (ONOO−). Peroxynitrite, a highly reactive species, can induce a proinflammatory posttranslational modification in cellular proteins known as tyrosine nitration. Increased nitrotyrosine in the endothelial cells is correlated with inflammatory molecule expression, cell death, and impaired endothelial function (reviewed in Ref. 36).

Premenopausal women are relatively protected against atherosclerosis compared with age-matched men. Higher circulating levels of the sex hormone estrogen are believed to play a protective role (46). However, this vasculo-protective role appears to be less apparent in women with diabetes, who have a high incidence of cardiovascular disease (27, 29, 32). Some of the estrogen effects on endothelium are now known to be modified or attenuated in the presence of oxidative stress, which may account for a lack of beneficial effects (47). Indeed, previous work from our group has demonstrated an altered estrogen receptor (ER)-α to ER-β ratio in endothelial cells exposed to both high glucose and estrogen (13).

NO, a major endothelium-derived vasodilator, exerts antioxidant, antiinflammatory, and antiapoptotic effects on the endothelium (18, 19, 28, 31). NO is synthesized from l-arginine during its conversion to l-citrulline, a process catalyzed by enzymes known as nitric oxide synthases (NOS). Three distinct isoforms of NOS are present in the body, namely, endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS) (34). Classically, eNOS has been considered the main source of NO in the vasculature, although there is increasing evidence that nNOS in the endothelium plays an important function (4, 9, 10, 17). Recently, we have demonstrated the presence of endothelial nNOS in rat mesenteric arteries where this enzyme contributes to a vasorelaxant role (33). The beneficial vascular effects of estrogen are believed to be largely mediated through increased endothelial NO synthesis (1, 2, 22). However, the specific contribution of the different NOS isoforms towards hyperglycemia-induced endothelial nitrotyrosine (a consequence of increased peroxynitrite) is incompletely understood. In addition, the molecular mechanisms underlying the regulation of NOS isoforms under hyperglycemia, especially their regulation (or the lack of it) by estrogen under such conditions, remain unknown.

Given this background, we hypothesized that high glucose conditions would alter the normal estrogen responses on NOS activity, NO generation, and subsequent peroxynitrite/nitrotyrosine formation in the vascular endothelium. We treated cultured human umbilical vein endothelial cells (HUVECs) with physiologically relevant doses of estrogen in the presence of normal or elevated concentrations of glucose to examine this hypothesis.

**Reagents.** Dulbecco’s phosphate-buffered saline (PBS), M199 medium with phenol red, porcine gelatin, and dithiothreitol (DTT) were all bought from Sigma Chemical (St. Louis, MO). Diaminofluorescein-FM (DAF-FM), M199 medium without phenol red, and fetal bovine serum (FBS) were obtained from Gibco/Invitrogen (Carlsbad, CA). Type 1 collagenase was purchased from Worthington Biochemical (Lakewood, NJ). Triton X-100 and endothelial cell growth...
supplement (ECGS) were both from VWR International (West Chester, PA). Dihydroethidium (DHE) and dihydrorhodamine (DHR) were purchased from Molecular Probes (Eugene, OR).

Endothelial cell culture and treatment. HUVECs were isolated from umbilical cords obtained from the Royal Alexandra Hospital in Edmonton, AB, Canada. Only umbilical cords from uncomplicated pregnancies (both male and female infants) were used. The protocol was approved by the University of Alberta Ethics Committee, and the investigation also conformed to the principles outlined in the Declaration of Helsinki. All subjects provided written informed consent before inclusion into this study. Briefly, the umbilical vein was first flushed with PBS to remove blood clots and then HUVECs were isolated out using a type 1 collagenase-containing buffer. The cells were grown in a humidified atmosphere at 37°C with 5% CO2-95% air in M199 medium with phenol red supplemented by 20% FBS as well as l-glutamine (GIBCO/Invitrogen), penicillin-streptomycin (Life Technologies, Gaithersburg, MD), and 1% ECGS. Cell culture medium contained physiological levels (5.5 mM) of glucose. Second passage cells were used for all experiments. HUVECs were chosen since these cells express both estrogen receptors (ER-α and ER-β) and are widely used as an in vitro model system to study the endothelium (13).

Confluent monolayers of second passage HUVECs were quiesced in a quiescing medium (Q-medium: phenol-free M199 media with 1% FBS and 1% penicillin-streptomycin) for 4 h before incubation with physiological (5.5 mM) or high (15.5 or 30.5 mM) concentrations of glucose and/or E2 (1–10 nM) for 3 or 24 h. The 3-h time point was chosen to detect the generation of short-lived species such as superoxide and peroxynitrite while the longer time point was used to examine nitrotyrosine, a long-lasting protein modification that indicates the “footprint” of peroxynitrite. An osmotic control, i.e., cells treated with mannitol similar to that described by Han et al. (25), had no effect on nitrotyrosine (Fig. 1C). To specifically examine the effects of high glucose on E2-mediated changes, E2 was added 1 h after addition of glucose since a 1-h incubation with high glucose is sufficient to increase ROS and reduce NO bioavailability as shown by Bagi et al. (5). Trypan blue staining demonstrated no significant alterations in HUVEC viability even after 24 h of treatment with high glucose, with or without E2 (data not shown).

Immunofluorescence. For epifluorescence studies (all fluororescent microscopy images except Fig. 4B), confluent HUVEC monolayers were fixed in 2% formalin, permeabilized with 0.1% Triton X-100, and immunostained using overnight incubation with a rabbit polyclonal antibody against nitrotyrosine (1:200; Chemicon, Temecula, CA) or mouse monoclonal antibodies against eNOS, iNOS, and nNOS (1:150; BD Biosciences, Mississauga, ON, Canada). Cells were treated with Alexa Fluor546 (red)-conjugated goat anti-rabbit and Alexa Fluor488 (green)-conjugated anti-mouse secondary antibodies (Molecular Probes) for 30 min in the dark. Nuclei were stained with the Hoechst 33342 nuclear dye from Molecular Probes (Molecular Probes, Mississauga, ON, Canada). Cells were treated with Alexa Fluor546 (red)-conjugated goat anti-rabbit and Alexa Fluor488 (green)-conjugated anti-mouse secondary antibodies (Molecular Probes) for 30 min in the dark. Nuclei were visualized under an Olympus IX81 fluorescence microscope (Carson Scientific Imaging Group; Markham, ON, Canada) attached to a CoolSNAP HQ2 camera (Photometrics, Tucson, AZ) using the Slidebook 2D, 3D Timelapse Imaging Software (Intelligent Imaging Innovations, Denver, CO). Because it was a monochrome camera, images from secondary antibodies labeled with Alexa488 were assigned green color, while those with Alexa488 were assigned red color for the purpose of demonstration. All images are magnified 100 times, i.e., images were taken using a (×10) dry objective. The background autofluorescence was subtracted from all images, so that cells treated with secondary antibody alone (i.e., without any primary antibody) appeared completely black (Fig. 1B).

We further validated the subcellular localization of nNOS in HUVECs by using confocal microscopy. HUVECs were grown on sodium bicarbonate-treated glass coverslips (1.5 mm thick), fixed, permeabilized, and immunostained with anti-nNOS antibody as described previously and imaged using a Leica SP5 laser-scanning confocal microscope (Leica Microsystems, Deerfield, IL) with a 60X water-immersion objective (i.e., 600 times magnification). Data were analyzed using the Leica Application Suite (LAS) software program (Leica Microsystems).

Peroxynitrite detection. Endothelial peroxynitrite was determined by DHR (excitation/emission wavelengths of 500/536 nm on reaction with peroxynitrite) staining (38). Confluent HUVEC monolayers were washed once and incubated for 30 min at room temperature with 25 μM DHR in Q-medium. After a 30-min incubation period, cells were washed once and fluorescence was visualized in a fluorescence microscope. For each data point, images from three randomly chosen fields were taken. The total fluorescence intensity and the number of cells in each field were noted, and the mean fluorescence intensity per cell (MFI/cell) was determined. Peroxynitrite generation was measured as fold increase in MFI/cell over the untreated control (5.5 mM glucose and no E2 or other reagent).

Superoxide detection. Endothelial superoxide generation was measured by staining with DHE similar to our previous work (13). DHE (excitation/emission wavelengths of 535/610 nm for oxidized product) is cell permeable and reacts with superoxide to yield ethidium, which binds to nuclear DNA and generates nuclear fluorescence (39). Briefly, HUVEC monolayers were washed once and incubated for 30 min at room temperature with 10 mM DHE in Q-medium. At the end of this 30 min incubation period, cells were washed once and fluorescence was visualized in a fluorescence microscope. For each data point, images from three randomly chosen fields were taken. The total fluorescence intensity and the number of cells in each field were noted and the mean fluorescence intensity per cell (MFI/cell) was determined as previously described (13). Superoxide generation was measured as fold increase in MFI/cell over the untreated control (5.5 mM glucose and no E2 or other reagent). This method for superoxide detection has been previously validated in our laboratory as the high glucose induced increase in ethidium (i.e., oxidized DHE) fluorescence was abolished by superoxide dismutase (13).

Nitric oxide detection. Endothelial NO generation was determined by DAF-FM (excitation/emission wavelengths of 495/515 nm on reaction with NO staining) (38). Confluent HUVEC monolayers were washed once and incubated for 30 min at room temperature with 5 μM DAF-FM in Q-medium. After a 30-min incubation period, cells were washed once and fluorescence was visualized in a fluorescence microscope. For each data point, images from three randomly chosen fields were taken. The total fluorescence intensity and the number of
Fig. 1. 17β-Estradiol (E2)-induced modulation of high glucose-induced nitrotyrosine and peroxynitrite in endothelial cells. A: human umbilical vein endothelial cells (HUVECs) were pretreated with elevated (15.5 or 30.5 mM) levels of glucose for 1 h before stimulation with E2 (1 or 10 nM) for 23 h. Cells were fixed, permeabilized, and immunostained for nitrotyrosine. A representative set of images from 4-7 independent experiments is shown. MFI, mean fluorescence intensity. Data are presented as means ± SE. B: HUVECs were either left untreated (Untr) or were treated with SIN-1 (1 mM) for 24 h before being fixed, permeabilized, and immunostained with an anti-nitrotyrosine antibody. A control experiment with secondary antibody (Sec Ab) alone is shown on the left. Representative images from 3 independent experiments are shown. C: HUVECs were treated with mannitol for 24 h before being fixed, permeabilized, and immunostained for nitrotyrosine as described previously. Representative images from 3 independent experiments are shown. D: HUVECs were pretreated with normal (5.5 mM) or elevated (15.5 or 30.5 mM) concentrations of glucose for 1 h before a 2-h incubation with E2 (1 or 10 nM). Cells were washed once, and intracellular peroxynitrite was determined by dihydrorhodamine (DHR) staining. A representative set of images from 5 independent experiments is shown. Data are presented as means ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the untreated control (5.5 mM glucose and no E2). Bar, 40 μm.
cells in each field were noted, and the mean fluorescence intensity per cell (MFI/cell) was determined. NO generation was measured as fold increase in MFI/Cell over the untreated control (5.5 mM glucose and no E2 or other reagent).

**Nitrate/nitrite estimation.** The amount of NO produced by the HUVECs on exposure to E2 or specific ER agonists was determined by estimating the total concentration of nitrate/nitrite in the cell-free supernatants using a commercially available Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI) based on a modified Griess reaction.

**Western blotting.** At the end of the specified incubation period, the HUVECs were lysed in boiling hot Laemmli’s buffer containing 0.2% Triton X-100 to prepare samples for Western blotting. Rapid cell lysis in this hot buffer causes immediate inactivation of cellular enzymes, thus precluding the use of inhibitors against phosphatases and/or proteases. This method of preparing endothelial protein lysates has been previously used by our group (13). Upon Western blotting, the protein bands were detected by a Fluor-S-Max multimager and quantified by densitometry using Quantity One software (Bio-Rad, Hercules, CA). Data from phospho-nNOS (antibodies from Abcam, Cambridge, UK) and phospho-eNOS (antibody from Cell Signaling Technology, Beverly, MA) bands were normalized to the corresponding total nNOS or total eNOS band, respectively. Samples derived from a particular umbilical cord were always run on the same gel. Cell lysates from untreated cells were loaded on every gel, and all data are expressed as fold increase over the corresponding untreated control (5.5 mM glucose and no E2 or other reagent). All antibodies were used at a final concentration of 1 μg/ml in TPBS (0.05% Tween 20 in phosphate-buffered saline).

**Statistics.** All data are presented as means ± SE from four to seven experiments. Each independent experiment was performed using cells obtained from a different umbilical cord. Multiple data groups were compared using one-way analysis of variance (ANOVA) with a Tukey’s post test. Two-way ANOVA was used where necessary to determine the interaction between two factors, with a Bonferroni’s post test to compare among different groups. Repeated measures were used where appropriate. P < 0.05 was taken as significant.
high glucose, rather than increased osmolarity, was required for nitrotyrosine. We next examined the effect of E2 on high glucose-induced peroxynitrite itself. Exposure to high glucose (15.5 or 30.5 mM) for 3 h increased intracellular DHR staining, indicating an increase in peroxynitrite generation. Interestingly, this DHR staining appeared to be localized to the cytoplasm, with no nuclear staining observed. Addition of E2 (1 or 10 nM) after 1 h of high glucose prevented the rise in DHR fluorescence (Fig. 1D). These data suggest an inhibitory effect of E2 on endothelial peroxynitrite generation under high glucose conditions.

E2 has no effect on high glucose-generated superoxide. High glucose conditions cause increased superoxide generation in endothelial cells, which can combine with basal NO levels to generate peroxynitrite, with consequent tyrosine nitration of cellular proteins. Since E2 attenuated high glucose-induced peroxynitrite, we then investigated E2 effects on endothelial superoxide. Incubation with high concentrations of glucose (15.5 or 30.5 mM) for 3 h increased DHE staining in endothelial cells, indicating increased superoxide generation (Fig. 2A). E2 (1 or 10 nM) added 1 h after addition of glucose did not prevent the rise in endothelial ethidium fluorescence under increased glucose concentrations (Fig. 2A), similar to previous findings from our laboratory (13). The osmotic control, mannitol, had no effect on changes in ethidium fluorescence (Fig. 2B).

E2 differentially regulates endothelial NO generation under high glucose. Since E2 had no effect on superoxide production, we next examined whether it could regulate the NO levels in high glucose-treated endothelial cells. E2 is known to activate eNOS, the major NO-producing enzyme in the endothelium under normal conditions (14). In addition, endothelial cells also express nNOS, another target of E2 in several different cell types (21, 24). However, the ability of E2 to regulate NO generation in high glucose-treated endothelium is not known. We found that high glucose alone had no significant effect on NO production in HUVECs as determined by DAF-FM staining. E2 had a significant effect on the DAF-FM fluorescence; it appeared to increase under normal glucose but decrease under high glucose (Fig. 3A). There was a highly significant interaction between glucose and E2, indicating that glucose concentrations altered the effects of E2 on NO generation. The sensitivity of this method for detecting intracellular NO was validated by treating HUVECs with different concentrations of the NO donor S-nitroso-N-acetyl penicillamine (SNAP, 0.1 mM and 1 mM), which caused a dose-dependent increase in DAF-FM fluorescence (Fig. 3B). In a similar vein, both E2 (10 nM) and high glucose

Fig. 3. E2 attenuates endothelial nitric oxide (NO) production in the presence of high glucose. A: HUVECs were pretreated with normal (5.5 mM) or elevated (15.5 or 30.5 mM) concentrations of glucose for 1 h before a 2-h incubation with E2 (1 or 10 nM). Cells were washed once, and intracellular NO was determined by diaminofluorescein-FM (DAF-FM) staining. A representative set of images from 5 independent experiments is shown. Data are presented as means ± SE. A 2-way ANOVA was used to determine the interaction between glucose concentrations and E2 levels. **P < 0.01 and ***P < 0.001. NS, not significant. #P < 0.05 compared with the corresponding data point under normal glucose (5.5 mM glucose and 10 nM E2). B: HUVECs were incubated with different concentrations of S-nitroso-N-acetyl penicillamine (SNAP) in presence of DAF-FM for 30 min before being washed and visualized. Representative images from 3 independent experiments are shown. C: HUVECs were treated with normal (5.5 mM) or high (30.5 mM) glucose for 1 h before 23-h incubation with E2 (1 or 10 nM). Cell-free supernatants were collected and analyzed for nitrate/nitrite. *P < 0.05 and **P < 0.01 compared with the untreated control (5.5 mM glucose and no E2). &&&P < 0.01 and &&&&P < 0.001 compared with high glucose (30.5 mM) alone. Bar, 40 μm.
(30.5 mM) individually increased the total nitrate/nitrite levels in endothelial culture supernatants, while E2 (1 or 10 nM) added 1 h after high glucose treatment significantly attenuated the rise in nitrate/nitrite concentrations (Fig. 3C).

**HUVECs express all three NOS isoforms.** Given the role of E2 in differentially regulating endothelial NO production under different glucose concentrations, we examined the molecular mechanisms underlying this novel process. To determine the role for one or more NOS isoforms, we first examined NOS expression in our HUVECs by immunostaining with anti-eNOS, anti-iNOS, and anti-nNOS antibodies. All these were mouse monoclonal antibodies used at identical concentrations (1:150) with the same secondary antibodies. We found that all three NOS isoforms were expressed in HUVECs (Fig. 4A), similar to previously published data from several different groups (4, 9, 16). Interestingly, nNOS demonstrated a prominent nuclear localization, similar to the findings by several other groups (43, 48), the significance of which in endothelial cells (such as on the regulation of protein expression) is currently being examined in our laboratory. We further confirmed the nuclear location of nNOS by confocal microscopy (Fig. 4B).

**nNOS is critical for high glucose-induced endothelial nitrotyrosine.** Next we studied the role of different NOS isoforms on high glucose-mediated tyrosine nitration in the endothelium. We used selective inhibitors against iNOS (1400W, 10 μM) and nNOS [N^ω-propyl-L-arginine (l-NPA), 2 μM]. HUVECs were treated with these inhibitors for 30 min before 24-h incubation with normal (5.5 mM) or high glucose (15.5 or 30.5 mM). l-NPA is a highly selective nNOS inhibitor that demonstrates ~3,150-fold and ~150-fold selectivity to nNOS compared with iNOS and eNOS, respectively (49). Several groups have used l-NPA at micromolar concentrations in different systems to demonstrate selective nNOS inhibition (3, 11). A recently published study from our group has shown that similar concentrations of l-NPA can indeed inhibit nNOS in the vasculature without affecting eNOS-dependent vascular functions (33). Ongoing work in our laboratory also suggests similar effects of 2 μM l-NPA compared with small interfering RNA-mediated downregulation of nNOS protein in HUVECs (data not shown). We found that nNOS inhibition almost completely abolished the high glucose-induced increase in endothelial nitrotyrosine, while iNOS inhibition had no effect (Fig. 5A). The degree of nitrotyrosine inhibition by l-NPA was comparable to that induced by the nonselective NOS blocker N^ω-nitro-L-arginine methyl ester (l-NAME; Fig. 5B), suggesting an essential role for endothelial nNOS in providing the NO involved in peroxynitrite (and hence, nitrotyrosine) generation under high glucose.

**nNOS phosphorylation is differentially regulated by E2 under normal and high glucose.** Finally, we examined the effects of E2 on nNOS activation status under various glucose concentrations. Similar to eNOS, nNOS activity is also regulated by phosphorylation at different sites. Two of these sites are well characterized; phosphorylation at Ser847 inactivates nNOS, while that at Ser1417 activates it. We treated HUVECs with various concentrations of glucose (5.5, 15.5, or 30.5 mM) for 1 h before 2 h of incubation with E2 (1 or 10 nM). While glucose alone had no effect on nNOS phosphorylation patterns, E2 had a significant effect which was further altered in the presence of different glucose levels. E2 under high glucose suppressed the phosphorylation at the activation-specific site Ser1417 (Fig. 6A) while increasing phosphorylation at the inactivation-specific site Ser847 (Fig. 6B).

To check whether these E2 effects were present even on longer periods of incubation, HUVECs were treated with various concentrations of glucose (5.5, 15.5, or 30.5 mM) for 1 h before a 23-h incubation with E2 (1 or 10 nM) for these experiments. Indeed, under high glucose conditions, we found a similar pattern of E2-mediated suppression of nNOS phosphorylation at the activating site (Ser1417) while the inactivating phospho-site (Ser847) remained unaffected (data not shown).

In comparison, E2 increased eNOS phosphorylation (activation-specific phosphorylation at Ser1177) under normal glucose while having no effects under high glucose levels, both at 3 and 24 h (Fig. 7). Together, these data suggest a novel role for E2 in regulating NOS activity through differential modulation of phosphorylation under normal and elevated glucose concentrations.
DISCUSSION

The key finding from this study is that estrogen effects on endothelial NO generation are differentially regulated under increasing concentrations of glucose. We demonstrated that E2 suppresses endothelial NO generation through inhibition of nNOS activity (as determined by changes in phosphorylation status) only under high (but not normal) glucose conditions. Suppression of NO generation by E2 contributed to a significant decrease in levels of both peroxynitrite and nitrotyrosine in high glucose-treated endothelium, without affecting superoxide levels.

Diabetes and estrogen have a relevant but complex interaction. Just over half of diabetes patients are women, and women with diabetes suffer much morbidity and mortality from cardiovascular diseases (32, 42). Gestational hypertension is another condition with concomitant high glucose and high estrogen levels in the circulation (42). Not only is this a major health problem by itself (affecting mostly younger women) but these women and their children are also at an increased risk of future cardiovascular morbidity (12). The normally vasculo-protective effects associated with the female sex appear to be absent in women with diabetes (27, 29, 32). Exogenous estradiol administration also produces limited vasodilatory effects in diabetic subjects compared with normal controls (30). These findings suggest alterations in the cellular pathways involved in mediating the beneficial estrogen effects on the vascular endothelium. Endothelial NO generation has been shown to be a major mechanism of beneficial estrogen effects on the vasculature (26, 45). High glucose conditions can potentially negate the effects of estrogen on vascular NO, either through reduced bioavailability, or, through direct modulation of NOS enzymes (15, 41).

Oxidative stress associated with diabetes is a key factor in endothelial disease and dysfunction. High glucose levels can
induce increased oxidative stress, with generation of superoxide. In combination with endogenous NO, superoxide produces peroxynitrite, which, in turn, leads to production of nitrotyrosine and activation of proapoptotic and proinflammatory pathways in the endothelium, in addition to reduced NO bioavailability (36, 41). While ROS generation by mitochondria and NADPH oxidase have been identified as primary sources of glucose-induced superoxide, the source of NO (for peroxynitrite formation) is less known (20, 35). Traditionally, eNOS has been described as the critical enzyme for endothelial NO production although endothelial cells express both eNOS and nNOS even under resting conditions (4, 17). However, even a short period (1 h or more) of exposure to high glucose conditions can lead to ROS-mediated uncoupling of eNOS, whereby eNOS yields superoxide instead of NO (20). The ROS-induced loss of tetrahydrobiopterin in the cytoplasm also plays a role in eNOS uncoupling under these conditions (37). Hence, there is much uncertainty about the origins of NO in high glucose-treated endothelial cells. Several studies have suggested a distinct role for nNOS compared with eNOS on vascular function and the regulation of inflammatory response (7, 10, 44). The reasons for this discrepancy are incompletely understood. While both

![Fig. 6. E2 differentially regulates nNOS phosphorylation under short-term (3 h) high glucose incubation. HUVECs were pretreated with normal (5.5 mM) or elevated (15.5 or 30.5 mM) concentrations of glucose for 1 h before a 2-h incubation with E2 (1 or 10 nM). Cells were lysed and immunoblotted with antibodies against the activation-specific (A) and inactivation-specific (B) phosphorylation sites of nNOS as well as a total nNOS antibody. Protein bands were quantified by densitometry. Data from phospho-nNOS (p-nNOS) bands were normalized to the corresponding total nNOS band. A representative set of images from 6 independent experiments is shown. Data are presented as means ± SE. A 2-way ANOVA was used to determine the interaction between glucose concentrations and E2 levels. *P < 0.05 and **P < 0.01. NS, not significant. #P < 0.05 compared with the corresponding data point under normal glucose (5.5 mM glucose and 10 nM E2).](http://ajpcell.physiology.org/)

![Fig. 7. E2 effects on eNOS phosphorylation under short-term (3 h) and long-term (24 h) high glucose incubation. HUVECs were pretreated with normal (5.5 mM) or elevated (15.5 or 30.5 mM) concentrations of glucose for 1 h before a 2-h (A) or 23-h (B) incubation with E2 (1 or 10 nM). Cells were lysed and immunoblotted with phospho-eNOS (p-eNOS) and total eNOS antibodies. Protein bands were quantified by densitometry. Data from p-eNOS bands were normalized to the corresponding total eNOS band. A representative set of images from 4 independent experiments is shown. Data are presented as means ± SE. *P < 0.05 and **P < 0.01 compared with the untreated control (5.5 mM glucose and no E2).](http://ajpcell.physiology.org/)
nNOS and eNOS generate NO, there might be differences in the duration, amount, and localization of NO generated, affecting the final result. The differing subcellular locations of distinct NOS isoforms can lead to different outcomes. Indeed, in cardiomyocytes, the localization of nNOS in the sarcomeram and eNOS in the caveolae appear to explain their different functional roles (6). Given the predominantly nuclear location of nNOS in human endothelial cells, it is likely to be relatively protected against uncoupling from increase in ROS generation (which is largely cytoplasmic, being produced by mitochondria, NADPH oxidase, and uncoupled eNOS). Indeed, our results show the presence of peroxynitrite limited to the cell cytoplasm, presumably a result of diffusion of NO from the nucleus and the cytoplasmic superoxide. Thus endothelial nNOS is likely to be a major source of NO in high glucose-induced increase in tyrosine nitration.

Estrogen can directly activate nNOS through modulation of its phosphorylation status. E2-mediated increased nNOS activity has been demonstrated in both vascular smooth muscle cells and neuronal cells (21, 24). However, the pathways leading to endothelial nNOS regulation have never been studied before. Our data suggest a novel regulation of nNOS involving both glucose and E2. Under normal glucose concentrations, E2 showed a trend towards increased nNOS activation, while, paradoxically, E2 suppressed nNOS activity under high glucose. Thus, glucose concentrations significantly altered the effects of E2 on endothelial nNOS and consequent NO generation. In a similar vein, E2 increased eNOS activation under normal glucose, while such responses were blunted under high glucose conditions. Despite the reductions in peroxynitrite and nitrotyrosine, it does not appear that E2 actually altered eNOS uncoupling, since the superoxide levels were unaffected under high glucose conditions, even after the addition of E2.

The significance of our findings goes beyond the attenuation of endothelial nitrotyrosine. While an E2-mediated reduction in endothelial peroxynitrite (and hence, nitrotyrosine) may be apparently beneficial, the physiological consequences of a reduced NO generating capacity can be far more serious. There is increasing evidence that NOS exerts a beneficial effect under a number of inflammatory pathologies. Endothelial nNOS activity can have antiinflammatory and antiatherogenic effects, while nNOS-induced hydrogen peroxide can contribute to lowering of blood pressure (7, 9, 40). Given the emerging role for nNOS in maintaining cardiovascular health, any impairment of nNOS activity by E2 under hyperglycemic conditions may actually worsen the cardiovascular outcome by making the vasculature more susceptible to inflammation and vasoconstriction despite offering some protection against glucose-induced peroxynitrite. Our findings suggest a novel role for glucose concentrations in modulating E2 responses on the endothelium, particularly on alterations in nNOS activity and NO production. Further research is needed for a better understanding of a complex system in which oxidative stress, nitrosative stress, inflammation, and cell injury all play important roles.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.C. and S.T.D. conception and design of the research; S.C. and C.C.C. performed the experiments; S.C. and C.C.C. analyzed the data; S.C. interpreted the results of the experiments; S.C. prepared the figures; S.C. drafted the manuscript; S.C. and S.T.D. edited and revised the manuscript; S.C., C.C.C., and S.T.D. approved the final version of the manuscript.

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