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High glucose-induced oxidative stress alters estrogen effects on ERα and ERβ in human endothelial cells: Reversal by AMPK activator

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ABSTRACT

Estrogen appears to protect against cardiovascular disease in pre-menopausal women. However, these protective effects are absent in women with diabetes. The hyperglycemia and consequent oxidative stress observed in diabetes cause endothelial dysfunction, but specific effects on endothelial estrogen responses are not known. In this study, we hypothesized that high glucose conditions would alter the regulation of the estrogen receptors (ERs), ERα and ERβ, in endothelial cells, possibly through increased oxidative stress. The role of the AMPK activator AICAR was examined on modulating the effects of high glucose. Cultured human endothelial cells were exposed to physiologically relevant doses of 17-β-estradiol (E2) for 24 h in presence of normal (5.5 mM) and high (30.5 mM) levels of glucose. Protein levels of estrogen receptors, ERα and ERβ, were measured through western blotting. Oxidative stress was measured by the dihydroethidium (DHE) assay for superoxide. Under normal glucose, E2 increased the levels of ERα relative ERβ; however, high glucose reversed the estrogen effects on endothelial ER expression. AMPK activation restored the physiological estrogen responses, likely through amelioration of oxidative stress. Control of oxidative stress by AMPK activation or anti-oxidants could restore normal estrogen responses even in presence of hyperglycemia.

1. Introduction

Diabetes mellitus is an increasingly prevalent health problem worldwide with 246 million people afflicted with this condition all over the world, just over half of whom (123.7 million) are women [1]. Cardiovascular diseases such as myocardial infarction and stroke are a major cause of excess morbidity and mortality in people with diabetes [2,3], with improvements in outcome observed on reducing hyperglycemia [4]. While women in the reproductive age group tend to have much lower rates of cardiovascular diseases compared to men [5], these beneficial effects are not evident in patients with diabetes [1,6–8]. The reasons for the absence of the vasculo-protective effects of the female sex are not clearly understood.

Estrogen is a key female sex hormone which exerts multiple anti-inflammatory and anti-oxidant effects on the vascular system [9–14]. Estrogen effects on the vasculature are mediated through two different estrogen receptors (ERs), namely, ERα and ERβ. Both receptor subtypes are expressed on the endothelium and appear to mediate similar as well as diverse roles on genomic and nongenomic estrogen signaling [15]. For example, ERα but not ERβ can mediate rapid endothelial nitric oxide generation through a nongenomic signaling pathway [16–19]. Indeed, long-term administration of ERα agonist Cpd1471 improved the endothelial dysfunction in a rat model of hypertension, at least partially through eNOS upregulation [20]. ERα knockout mice also lack the protective effects of estrogen supplementation on myocardial ischemia-reperfusion injury [21]. Thus, ERα appears to mediate many of the vasoprotective effects of estrogen on the endothelium in various experimental studies. Estrogen itself can regulate the expression of ERα and ERβ, increasing the former and decreasing the latter, in ovine fetal pulmonary endothelial cells [22]. It is not known whether estrogen receptor expression patterns in the cardiovascular system are altered in patients with diabetes.

High glucose concentrations as observed in diabetes induces oxidative stress in the vascular endothelium, generating an excess of superoxide radicals which may lead to formation of other free radicals such as peroxynitrite and hydrogen peroxide [23,24]. Increased superoxide is associated with endothelial dysfunction and the development of a pro-inflammatory phenotype, which can predispose the vasculature towards atherosclerotic changes [25–28]. Protein levels of the estrogen receptors, ERα and ERβ, can be differentially regulated under increased oxidative stress as

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shown by *in vitro* studies several cell lines (including endothelial cell line E304) [29]. However, the effects of hyperglycemia on regulation of estrogen receptors in the human endothelium are not known.

In recent years, the 5′-AMP activated kinase (AMPK) pathway has been identified as a potential target of anti-diabetic therapies [30,31]. AMPK is a cellular protective mechanism that can exert anti-oxidant, anti-inflammatory and anti-apoptotic effects on the vascular endothelial cells [32,33]. The pharmacological AMPK activator 5-aminoimidazole 4-carboxamide ribonucleoside (AICAR) has been shown to reduce high glucose-induced ROS generation in cultured endothelial cells and improve insulin sensitivity in an animal model of diabetes [34,35]. In addition, metformin, a commonly used anti-diabetic agent can activate AMPK which may account for its ability to reduce morbidity and mortality from cardiovascular diseases above and beyond its effects on lowering blood glucose [36,37]. However, the role of AMPK activation on restoring the protective estrogen effects on the vascular endothelium remains unknown.

Given the pro-oxidant effects of hyperglycemia and the ability of AMPK activation to ameliorate it, we hypothesized that high glucose conditions would alter the estrogen-mediated regulation of ERα and ERβ levels in endothelial cells and concomitant AMPK activation would restore the normal estrogenic responses. Our results suggest that high glucose-induced ROS generation indeed reverses the estrogen effects on endothelial ER expression, and the AMPK activator AICAR prevents such changes, likely through inhibition of superoxide generation.

2. Materials and methods

2.1. Reagents

Dulbecco's phosphate buffered saline (PBS), M199 medium with phenol red, porcine gelatin, cell culture tested D-glucose, AICAR, polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) and cyclodextrin-encapsulated 17-β-estradiol (E2) were all bought from Sigma Chemical Co. (St. Louis, MO). 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 Corporation (Lakewood, NJ). Triton-X-100 and endothelial cell growth supplement (ECGS) were both from VWR International (West Chester, PA). Dihydroethidium (DHE) was purchased from Molecular Probes (Eugene, OR).

2.2. Antibodies

Rabbit polyclonal primary antibodies against ERα and ERβ were both obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and used at a concentration of 1 μg/ml for western blots. The α-Tubulin antibody was from Abcam (Cambridge, MA) and used at 0.4 μg/ml. Goat anti-rabbit HRP-conjugated secondary antibody was purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA) and used at 1:5000 dilutions.

2.3. Endothelial cell culture and treatment

Human umbilical vein endothelial cells (HUVEC) were used as a model system as these are a readily available source of cultured endothelial cells that have been well characterized and widely used to study inflammation and oxidative stress in the vasculature. The protocols were approved by the University of Alberta Ethics Committee and the studies were conducted according to the principles of the Declarations of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects, Revised November 13, 2001, effective December 13, 2001. All subjects provided informed consent prior to inclusion in this study. HUVECs were isolated from umbilical cords obtained from the Royal Alexandra Hospital in Edmonton, AB. Briefly, the umbilical vein was first flushed with PBS to remove blood clots and then HUVECs were isolated out using a buffer containing 125 units/ml of type 1 collagenase. The cells were grown in a humidified atmosphere at 37 °C with 5% CO_2/95% air in M199 medium with phenol red supplemented by 20% FBS as well as L-glutamine (Gibco/Invitrogen), penicillin–streptomycin (Life Technologies) and 1% ECGS. Cell culture medium contained physiological levels (5.5 mM) of glucose. Second passage cells were used for all experiments.

On the day of the experiment, confluent monolayers (at 80–90% confluence) of second passage HUVEC were quiesced in a quiescing medium (phenol-free M199 media with 1% FBS and 1% penicillin–streptomycin) for 4 h prior to starting the actual experiment. Cells were treated with physiological (5.5 mM) or high (30.5 mM) concentration of glucose and/or E2 (1–20 nM) for 24 h. To specifically examine the effects of high glucose on E2-mediated changes, glucose was added 1 h prior to the addition of E2. 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. Trypan Blue staining demonstrated no significant alterations in HUVEC viability after 24 h treatment with high glucose, with or without E2 (data not shown).

2.4. Western blotting

Western blotting was performed on the HUVEC lysates as described before [38]. The protein bands were detected by a Fluor-S-Max multiimager and quantified by densitometry using Quantity One software (Bio-Rad, Hercules, CA). Data were normalized by reprobing the membrane with an antibody against α-Tubulin which was used as a loading control. Samples generated from a particular umbilical cord were run on the same gel. Cell lysates from untreated cells were loaded on every gel and all data were expressed as fold increase over the corresponding untreated control (5.5 mM glucose and no E2 or other reagent).

2.5. Superoxide detection assay

Endothelial superoxide generation was measured by staining with dihydroethidium (DHE). DHE 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 μM of DHE in the quiescing 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 similar to Dyugovskaya and coworkers [40]. Superoxide generation was measured as fold increase in MFI/cell over the untreated control (5.5 mM glucose and no E2 or other reagent).

2.6. Statistics

All data presented are mean ± SEM of between four and seven independent experiments using HUVECs isolated from different umbilical cords. All data are expressed as fold change over the untreated control (5.5 mM glucose and no E2 or other reagent). One way analysis of variance (ANOVA) with an appropriate post-test was used for determination of statistical significance. Dunnett's post-test was used for western blot data (except Fig. 3A) to compare the E2-treated groups to the corresponding E2-free control. Tukey's post-test for comparisons among multiple groups was used for the

superoxide data. A two way ANOVA was performed to determine the interaction between glucose concentrations and estrogen levels on ER expression. Repeated measures test was used wherever applicable. $P < 0.05$ was taken as significant.

3. Results

3.1. Effects of glucose on E2-mediated ERα regulation

Varying levels of glucose alone had no effect on protein levels of endothelial ERα (data not shown). Under normal glucose levels (5.5 mM), physiologically relevant doses (1–20 nM) of E2 increased endothelial ERα levels (Fig. 1A), which is commensurate with previously published findings [22]. Interestingly, under high (30.5 mM) glucose concentrations, exogenous E2 administration decreased ERα levels (Fig. 1B). Thus, the ability of E2 to increase ERα in the endothelium under physiological conditions was reversed under increased glucose concentrations. A two way ANOVA test showed a statistically significant interaction between glucose concentration and estrogen levels on expression of ERα, suggesting an interaction between these two different factors on the regulation of endothelial ERα expression. To exclude the possibility of osmotic changes affecting responses to high glucose, we used 25 mM mannitol (in culture medium containing 5.5 mM glucose) as an osmotic control similar to Han et al. [41]. Mannitol had no effect on the E2 responses (Fig. 1C), suggesting the effects of high glucose were not due to increased osmolarity alone. These data show a role for high glucose in altering E2-mediated effects on endothelial ERα levels.

3.2. Effects of glucose on E2-mediated ERβ regulation

As in the case of ERα, high glucose alone did not significantly change endothelial ERβ levels (data not shown). In contrast to ERα, E2 (1–20 nM) did not significantly alter ERβ levels under physiological glucose (Fig. 2A). Surprisingly, E2 increased ERβ levels under high (30.5 mM) glucose concentrations (Fig. 2B). Unlike the case of ERα, a two way ANOVA did not show any statistically significant interaction between glucose and estrogen concentrations on ERβ expression. Mannitol, used as an osmotic control, had no effect on

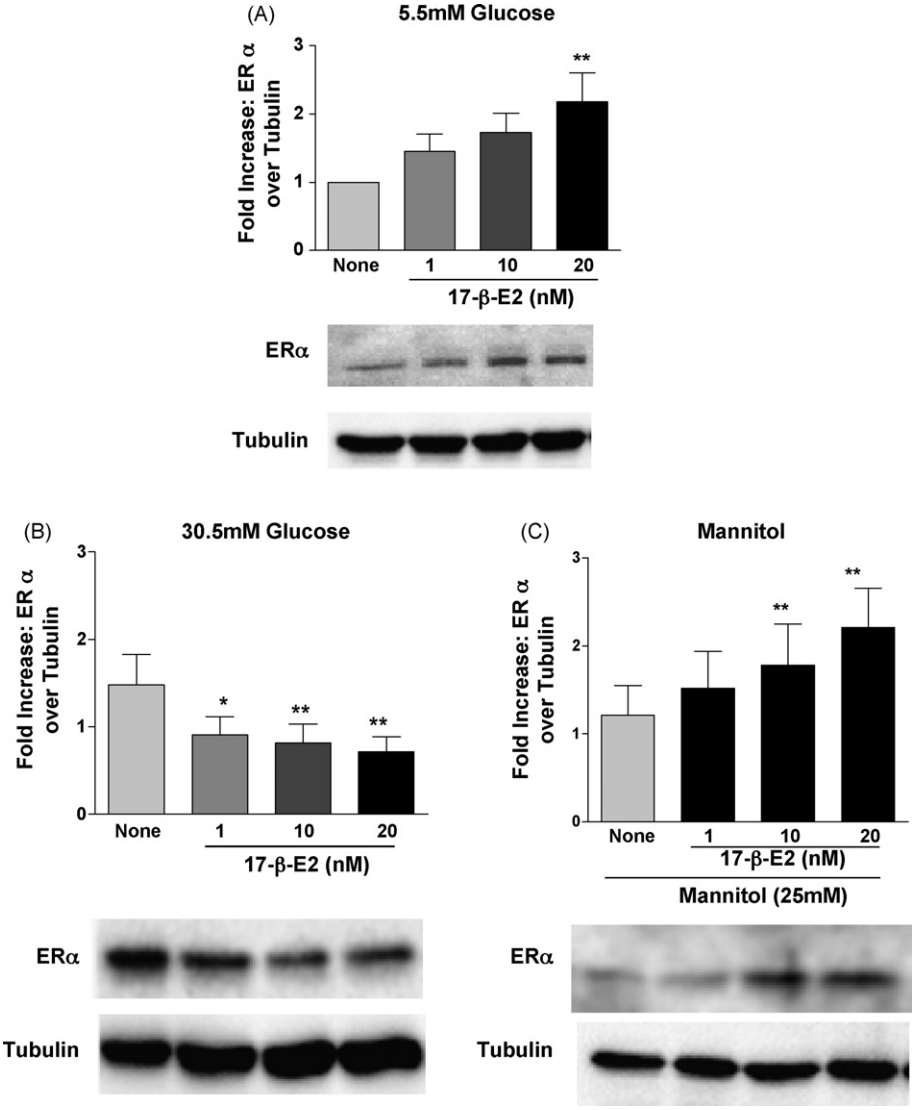

Fig. 1. Effects of glucose concentrations on E2-mediated changes in endothelial ERα levels. Confluent HUVEC monolayers were pre-treated for 1 h with physiological (5.5 mM, A) or high (30.5 mM, B) levels of glucose prior to 24 h incubation with different concentrations of E2. Mannitol (25 mM, C) was used instead of high glucose to control for osmolarity. ERα levels are expressed as fold increase over the untreated control. Representative western blots are shown. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively.

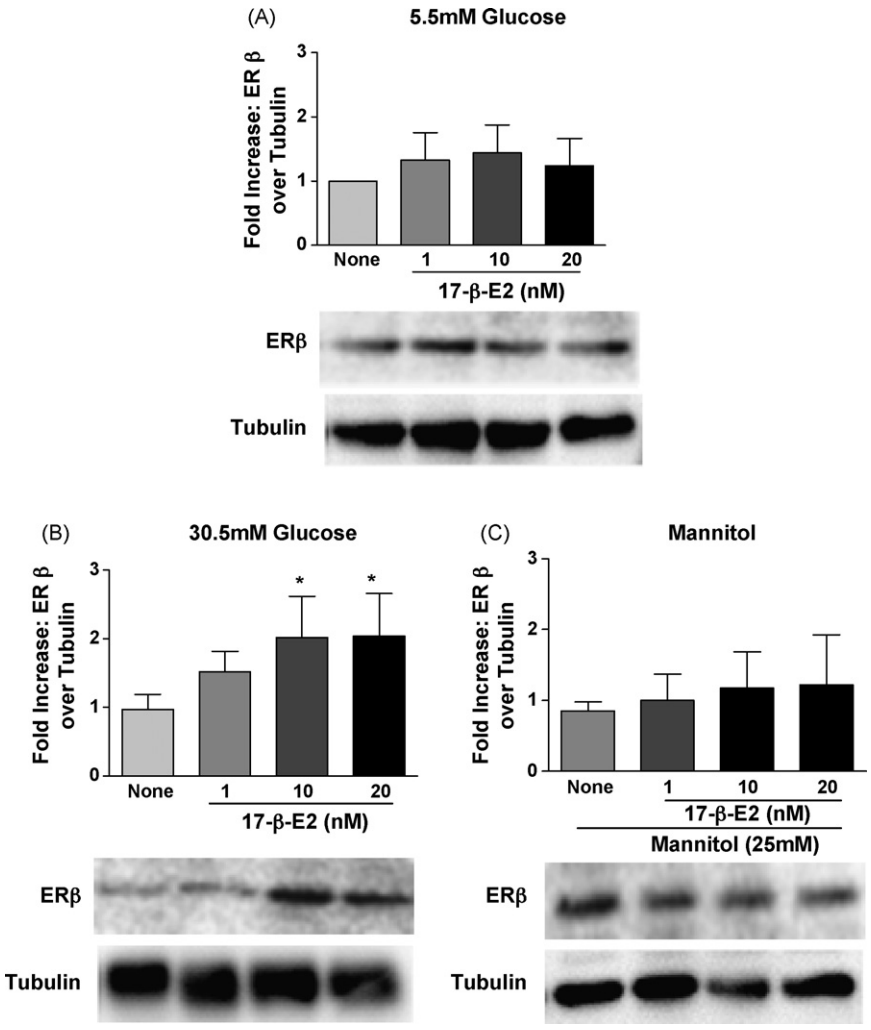

Fig. 2. Effects of glucose concentrations on E2-mediated changes in endothelial ERβ levels. Confluent HUVEC monolayers were pre-treated for 1 h with physiological (5.5 mM, A) or high (30.5 mM, B) levels of glucose prior to a 24 h incubation with different concentrations of E2. Mannitol (25 mM, C) was used to control for osmolarity. ERβ levels are expressed as fold increase over the untreated control. Representative western blots are shown. * indicates $P < 0.05$.

the E2 responses (Fig. 2C), suggesting the effects of high glucose were not due to increased osmolarity alone. These data show a role for high glucose in altering E2-mediated effects on endothelial ERβ levels.

In summary, the effects of E2 on the relative levels of ERα and ERβ were reversed under high glucose compared to a physiological level of glucose.

3.3. Role of AICAR and high glucose in modulating E2 effects on ERα and ERβ

Next we examined the role of the AMPK activator AICAR on E2 responses altered by high (30.5 mM) glucose levels. We found that a 24 h incubation with AICAR alone increased protein levels of ERα under physiological glucose levels without affecting ERβ. The significance of this finding is not clear at this point; although it is possible that AICAR may improve the ERα-mediated vasoprotective effects. In contrast, a 24 h treatment with AICAR under high glucose conditions did not alter either ERα or ERβ levels (Fig. 3A and B). However, prior administration of AICAR (for 60 min) prevented the effects of high glucose on E2 regulation of both ERα and ERβ levels in the endothelium (Fig. 3C and D). These data suggest that the AMPK pathway can ameliorate the high glucose-induced alterations on regulation of endothelial ERs.

3.4. Effects of AICAR on high glucose-mediated superoxide generation

We first demonstrated that treatment of HUVECs with AICAR caused increased phosphorylation (and hence, activation) of AMPK as well as that of its downstream target acetyl CoA carboxylase (ACC). AMPK activation by AICAR has been previously shown to attenuate the high glucose-induced ROS generation in endothelial cells [34]. We found that exposure to high glucose concentrations (30.5 mM) generated increased levels of superoxide ions in endothelial cells, which were completely abrogated by prior treatment with AICAR for 60 min, an effect that was prevented by co-administration of the AMPK inhibitor compound C (Fig. 4). Subsequent administration of E2, 60 min after the addition of high glucose did not alter the anti-oxidant effects of AICAR pretreatment (Fig. 4), while E2 alone had no effect.

3.5. Role of superoxide in mediating effects of high glucose on E2 regulation of ERα and ERβ levels

To demonstrate that the effects of high glucose concentration were actually due to increased superoxide generation, we used a cell permeable superoxide scavenger PEG-SOD for our final set of experiments. We found that prior treatment with PEG-SOD com-

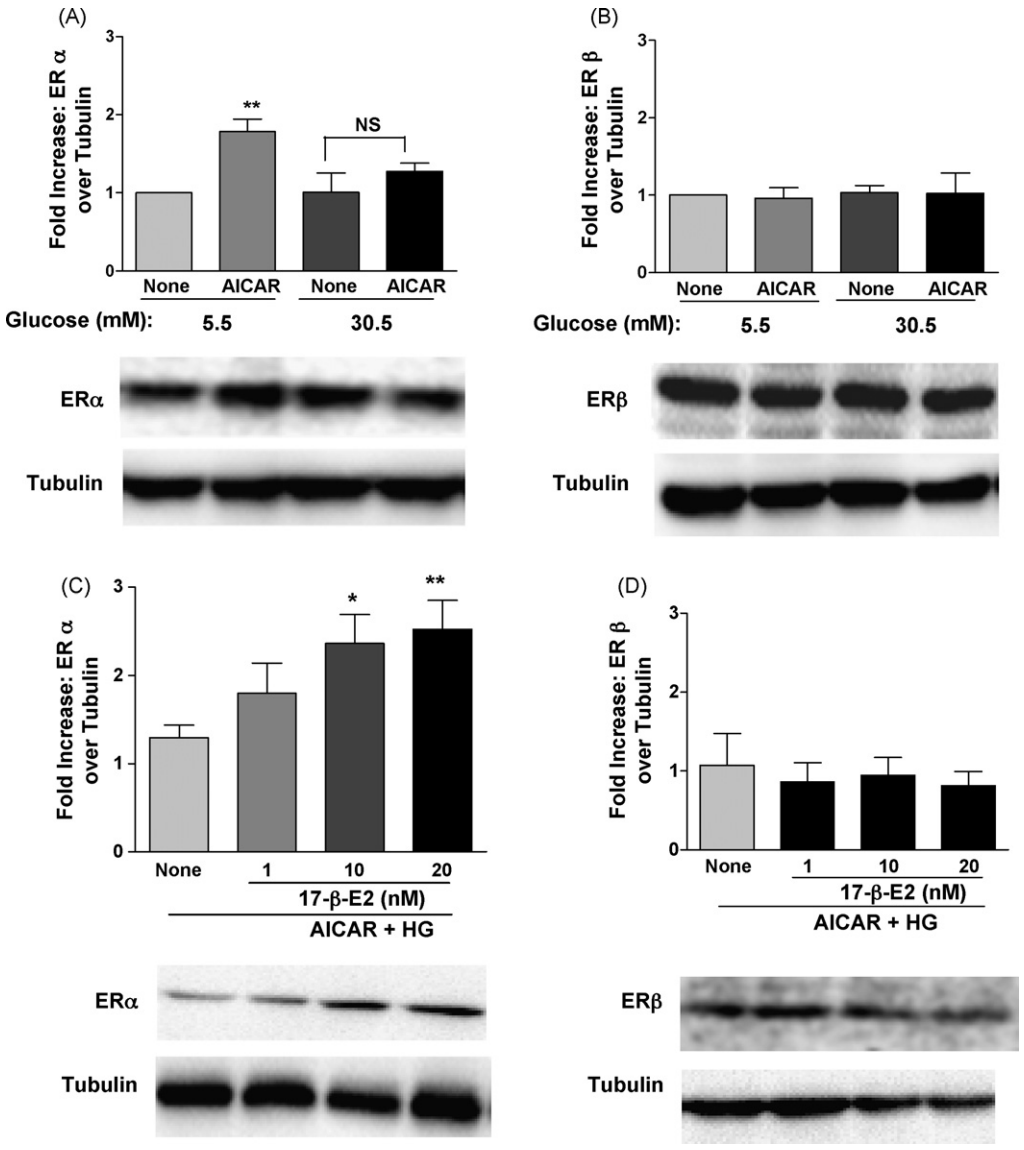

Fig. 3. Effects of AICAR on glucose and E2 effects on endothelial ERα and ERβ levels. (A) and (B) show the ERα and ERβ levels in HUVEC treated with AICAR (1 mM) for 1 h prior to a 24 h incubation with varying glucose concentrations. (C) and (D) show the combined effects of pre-treatment with AICAR (1 mM) and high glucose (HG, 30.5 mM) on subsequent E2 effects on endothelial ERα and ERβ levels. Representative western blots are shown. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively. NS means not significant.

pletely blocked high glucose-mediated oxidative stress (Fig. 5A). In addition, the superoxide scavenger restored the effects on E2 on ERα and ERβ levels in the presence of high glucose (Fig. 5B and C), in contrast to that observed with high glucose alone (Figs. 1B and 2B). These data suggest that high glucose-mediated superoxide generation alters the E2 effects on endothelial ERα and ERβ levels and abrogation of this superoxide-induced oxidative stress can restore the normal E2 responses.

4. Discussion

In this paper, we have shown a role for high glucose-induced oxidative stress in altering the estrogen-mediated regulation of ERα and ERβ expression in human endothelial cells. Under physiological glucose levels, estrogen increased ERα without significantly affecting ERβ levels, causing an increase in the relative concentrations of ERα to ERβ. These results are similar to those obtained by Ihionkhan et al. [22], although we did not observe an estrogen-induced decrease in ERβ, possibly due to species difference (human vs. ovine) and/or a shorter incubation period (24 h vs. 48 h). However, under high glucose conditions, we observed that estrogen decreased ERα and increased ERβ, leading to a decrease in the relative levels of ERα to ERβ. Thus, high glucose appears to reverse the estrogen effects on relative expression of its receptors. We also found a critical role for oxidative stress, especially the increase in superoxide ion levels, on this process. To the best of our knowledge this is the first time that glucose concentrations have been shown to modulate estrogen responses in the endothelium.

The protective estrogen effects on the vasculature are significantly diminished in patients with diabetes [7]. Indeed, while long-term estrogen supplementation improves endothelial function in normal women, such effects are totally absent in vessels from women with diabetes, suggesting a loss of the estrogen-mediated protection [42]. It is likely that differences in ER expression may explain some of these effects. While some overlap exists between the roles for these two ERs, distinct pathways mediated through individual ERs are increasingly becoming obvious, with a specific beneficial role for ERα on the vasculature [15]. Our findings suggest that high glucose reverses the increase in ERα-to-ERβ level induced by estrogen under physiological conditions. This is a possible mech-

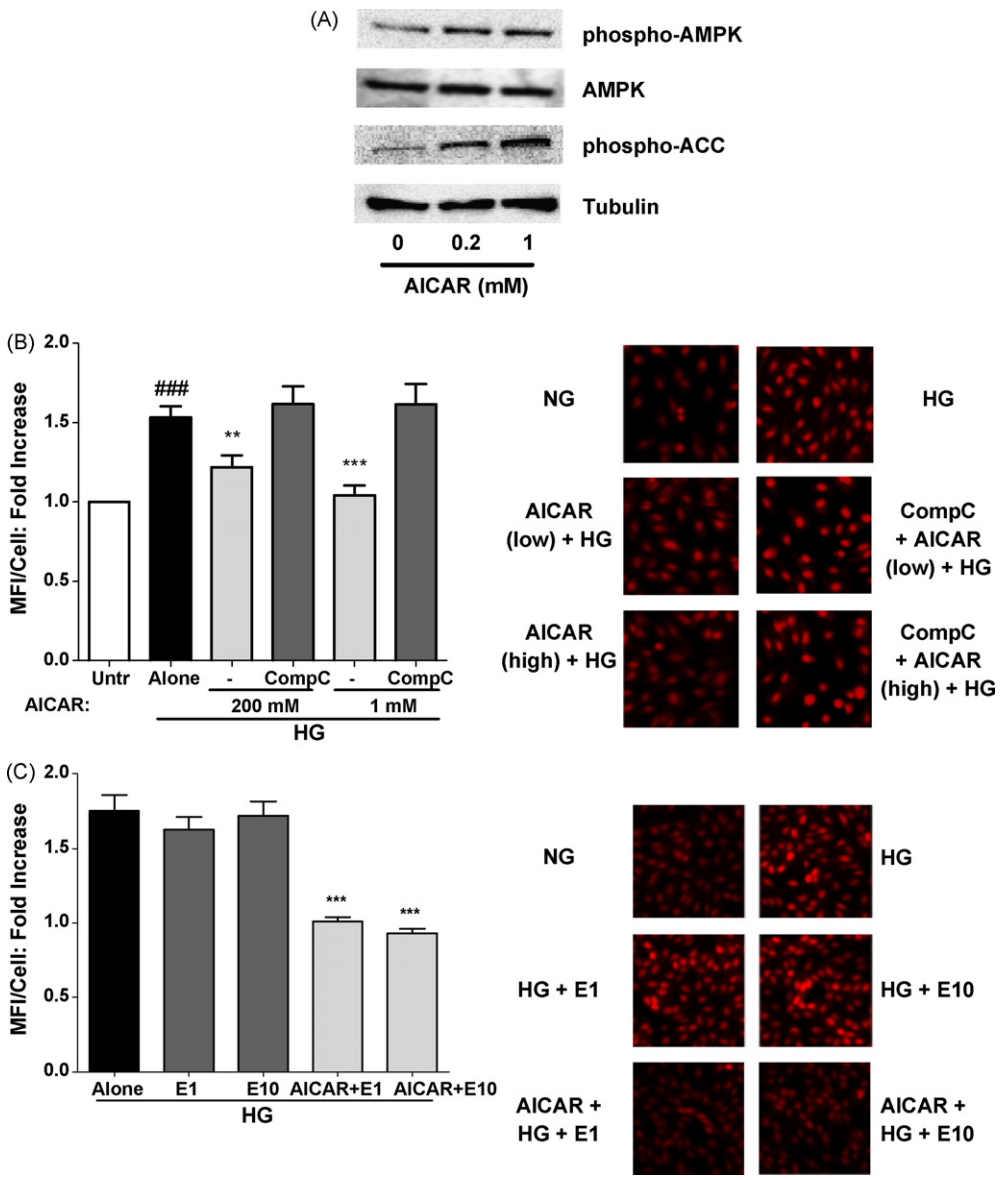

Fig. 4. Effects of AICAR, high glucose and E2 on endothelial superoxide generation. (A) Confluent HUVEC monolayers were treated with different concentrations of AICAR for 30 min. Representative blots of phospho-AMPK, total AMPK, phospho-ACC and Tubulin are shown. (B) HUVECs were treated for 3 h with normal (5.5 mM, NG) or high (30.5 mM, HG) glucose with or without pre-treatment with high (1 mM) or low (200 μM) concentrations of AICAR with/without AMPK inhibitor compound C (Comp C, 10 μM). (C) HUVECs were treated for 3 h with high (30.5 mM) glucose with or without 1 h pre-treatment with 1 mM AICAR and a 2 h treatment with 1 nM (E1) or 10 nM (E10) E2. Superoxide was measured using DHE assay. A representative set of images are shown. Data calculated as MFI/cell and expressed as fold increase over the untreated control. ### indicates $P < 0.001$ as compared to control; ** and *** indicate $P < 0.01$ and $P < 0.001$, respectively, compared to high glucose alone.

anism by which the vasculo-protective effects of estrogen may be abrogated under high glucose conditions. We have now shown a role for AMPK activation and superoxide scavenging on the prevention of such potentially harmful effects of high glucose on the endothelium.

As noted before, increased oxidative stress plays a key role the pathogenesis of cardiovascular complications in diabetes [43]. Reduction of oxidative stress through anti-diabetic drugs such as Gliclazide has been shown to improve cardiovascular parameters in diabetes, although the effect on responses to sex steroids remains unknown [44]. Although oxidative stress by itself was shown to upregulate ERβ but not ERα by Tamir et al., we did not observe any significant changes in ER levels due to high glucose alone. This may reflect the use of an exogenous and potentially stronger oxidant by Tamir et al. [29], vs. a more physiological measure (high glucose in culture medium for 24 h) used in this study. Pharmacological AMPK activators and other anti-oxidants may protect against the deleterious effects on uncontrolled ROS generation in the vasculature of persons with diabetes [34,45]. We found that the AMPK activator AICAR completely abrogated the effects of high glucose on estrogen regulation of ERα and ERβ. Similar effects on ER expression were observed using a cell permeable form of superoxide dismutase in the presence of exogenous estrogen. Such findings further support a critical role for superoxide-mediated oxidative stress as the critical factor in altering normal estrogen responses on the vasculature. Similarly, increased oxidative stress is observed in the aging vasculature, another instance where the normal beneficial role of estrogen appears to be abrogated [46]. Further investigations on the regulation of ER expressions in aging might be an interesting direction for our research.

The mechanisms linking oxidative stress with estrogen regulation of its own receptors are not clear yet. Recently, high glucose concentrations similar to those in our study has been shown to reduce S-nitrosylation of various proteins in endothelial cells in a

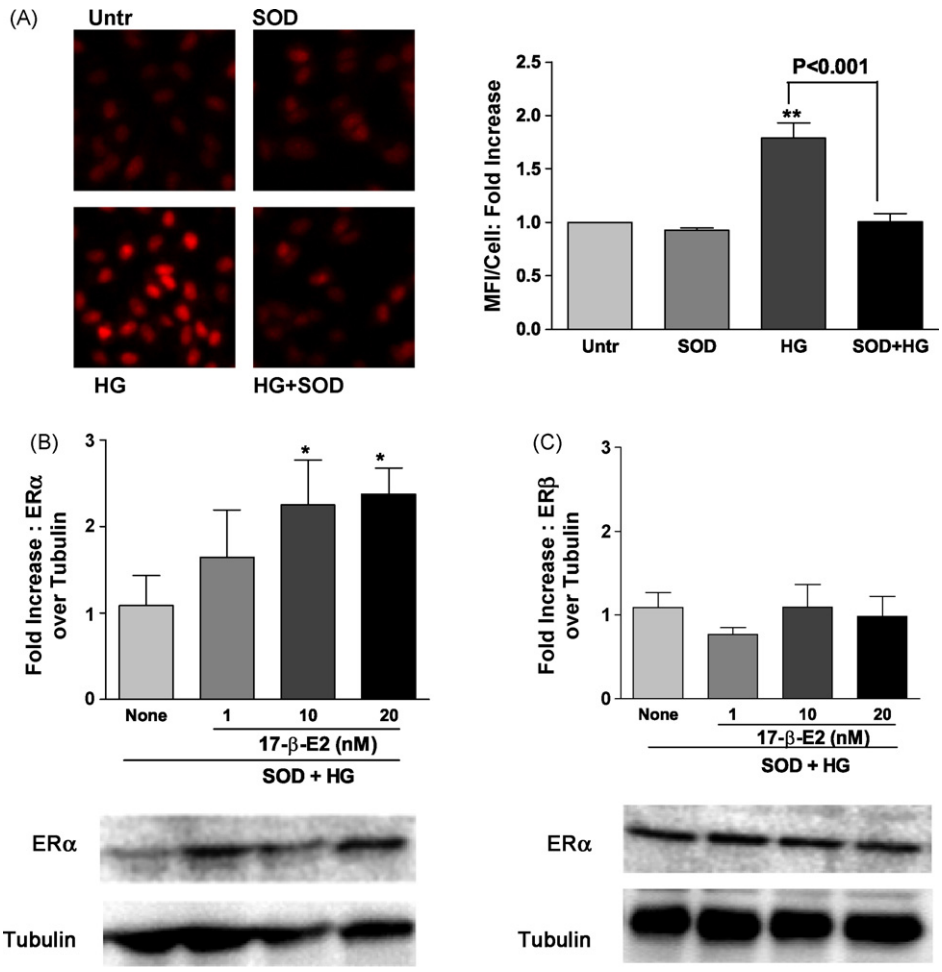

Fig. 5. Effects of superoxide scavenger PEG-SOD on E2 and high glucose-mediated changes on endothelial ER expression. (A) shows the ability of cell permeable SOD to successfully scavenge superoxide. Confluent HUVEC monolayers were treated for 3 h with normal (Untr, 5.5 mM) or high (HG, 30.5 mM) levels of glucose with or without 30 min pre-treatment with PEG-SOD (100 units/ml). Superoxide was detected by DHE assay as mentioned before. HUVECs pre-treated with PEG-SOD and high glucose (30.5 mM) for 1 h were treated with different concentrations of E2 for 24 h. ERα (B) and ERβ (C) levels were determined by western blot and quantified. Representative western blots are shown. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively.

ROS-dependent manner [47]. Estrogen can induce S-nitrosylation of ERα, a process which improves the nongenomic signaling at the expense of its classical genomic functions which may alter the regulation of ER expression [48]. Future studies need to be undertaken in this direction. There has been a greater appreciation of the role of anti-oxidants and AMPK activators in the treatment of diabetes and its complications in recent years. Our study suggests a novel role for these established anti-diabetic drugs on restoring normal estrogen responses in the vascular endothelium, which may have therapeutic implications for the management of women with diabetes.

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