High glucose abolishes the antiproliferative effect of 17β-estradiol in human vascular smooth muscle cells

SHANHONG LING,1 PETER J. LITTLE,2 MARO R. I. WILLIAMS,1 AOZHI DAI,1 KAZUHIKO HASHIMURA,1 JUN-PING LIU,3 PAUL A. KOMESAROFF,1,6 AND KRISHNANKUTTY SUDHIR1*

1Hormones and the Vasculature Laboratory, 2Cell Biology of Diabetes Laboratory, and 3Molecular Signaling Laboratory, Baker Medical Research Institute, Melbourne, Victoria 8008, Australia

Received 9 March 2001; accepted in final form 14 November 2001

Ling, Shanhong, Peter J. Little, Maro R. I. Williams, Aoizhi Dai, Kazuhiko Hashimura, Jun-Ping Liu, Paul A. Komesaroff, and Krishnankutty Sudhir. High glucose abolishes the antiproliferative effect of 17β-estradiol in human vascular smooth muscle cells. Am J Physiol Endocrinol Metab 282: E746–E751, 2002. First published December 4, 2001; 10.1152/ajpendo.00111.2001.—We examined the effects of 17β-estradiol (E2) on human vascular smooth muscle cell (VSMC) proliferation under normal (5 mmol/l) and high (25 mmol/l) glucose concentrations. Platelet-derived growth factor (PDGF) BB (20 ng/ml)-induced increases in DNA synthesis and proliferation were greater in high than normal glucose concentrations; the difference in DNA synthesis was abolished by a protein kinase C (PKC)-β inhibitor. In high glucose, the inhibitory effect of E2 on DNA synthesis and proliferation was abolished but was restored in the presence of the PKC-β inhibitor LY-379196. Thus high glucose enhances human VSMC proliferation and attenuates the antiproliferative effect of E2 in VSMC via activation of PKC-β.

METHODS

Cell culture. VSMC were prepared from internal mammary arteries of two women with coronary artery disease. The patients had hypertension and hypercholesterolemia, but no diabetes, and one of the patients had had a previous myocardial infarction. The internal mammary artery segments were obtained at the time of coronary artery bypass grafting, and VSMC were harvested by an explant technique (18). In brief, segments of internal mammary artery were cut out and placed into ice-cold Dulbecco’s modified Eagle’s medium (DMEM). All external fat and connective tissue were cleaned from the vessel under microscope. The outer membrane was torn off carefully, the vessel was cut longitudinally, and the endothelial layer was removed by scraping with forceps. Small strips of media were peeled off, transferred to 60-mm dishes anchored under 9% CO2, and incubated in normal glucose (5 mmol/l glucose) DMEM in the presence of 10% fetal bovine serum (FBS) in an incubator of 5% CO2 in air at 37°C. Culture media were changed every three days, and after ~1 wk, VSMC were.

It is currently accepted that premenopausal women have relative protection from cardiovascular disease, compared with men and postmenopausal women, probably due to the protective effects of estrogen (7, 21). However, premenopausal women with diabetes mellitus lose this gender-based cardiovascular protection (3, 16), suggesting that hyperglycemia possibly overcomes some of the beneficial effects of sex steroids. To date, little is known of the cellular and subcellular interactions between the signaling pathways associated with hyperglycemia and sex hormones in the vasculature.

Several lines of evidence suggest that estrogens inhibit vascular smooth muscle cell (VSMC) proliferation (14, 19, 23), whereas hyperglycemia stimulates VSMC growth (24, 26). Because VSMC proliferation is an important cellular mechanism in the development of atherosclerosis (17), an interaction between estrogens and glucose-related signaling pathways in regulating VSMC proliferation is possible. We hypothesized that high glucose concentrations might attenuate the antiproliferative effect of estrogens. In the present study, we examined the antiproliferative effects of 17β-estradiol (E2) under high (25 mmol/l glucose) and normal (5 mmol/l glucose) glucose concentrations on human internal mammary artery smooth muscle cells. We found that E2 inhibits VSMC proliferation in a dose-dependent manner, but its antiproliferative effect is abolished by high glucose concentrations through a mechanism dependent on the activation of protein kinase C (PKC)-β.
observed migrating from the pieces and growing on the dishes. Cells were grown to near confluence and passaged, and the presence of smooth muscle α-actin was confirmed by immunofluorescence staining and Western blot analysis as a marker of VSMC. Cells from the first patient were used at passages 6–7, and cells from the second patient were used at passages 11–15.

**Estrogen receptor studies.** Estrogen receptor (ER) density in these VSMC was determined by radioligand binding assay. Confluent monolayers of cells on 6-well plates (~10^6 cells/well) were incubated with [^3H]estradiol (0.31–5 nmol/l) and nonradioactive diethylstilbestrol (1 μmol/l) for 90 min. Cells were washed twice with Dulbecco’s PBS (D-PBS), scraped in the presence of 1.5 ml/well of 0.1% Triton-acetic acid, and put into scintillation vials with 5 ml of Scintillation Liquid Instagel (Bio-Rad, Sydney, NSW, Australia) for counting (5 min/vial) in a β-counter. Functional ER studies were carried out using the pure ER antagonist ICI-182780 (Tocris Cookson, Bristol, UK).

**Experimental protocol.** Cells were seeded in culture plates, grown to 90–95% confluence, and growth-arrested in serum-free DMEM with normal-glucose medium (5 mmol/l glucose, with 20 mmol/l mannose for control of osmolarity) or high-glucose medium (25 mmol/l glucose) for 48 h. Cell proliferation was induced with PDGF-BB (20 ng/ml), and effects of E2 were detected by preincubation with the hormone for 1 h before PDGF-BB stimulation.

**Assays of cell proliferation.** DNA synthesis was determined by a [^3H]thymidine incorporation assay. Cells in 24-well plates were incubated with 1 μCi/well of [^3H]thymidine during the last 3 h of PDGF-BB treatment, washed three times with ice-cold D-PBS, incubated with ice-cold 0.2 N HClO₄ (1 ml/well) on ice for 30 min, washed (0.5 ml/well, 3 times) with 0.2 N HClO₄, incubated with 0.5 ml/well of 0.2 N NaOH at 37°C for 1 h, and neutralized with 0.2 ml/well of 6% acetic acid. Contents of the wells were transferred into scintillation vials with 3 ml of Instagel and counted for 2 min per vial in a β-counter. Cell number was measured by an automatic cell counter (S.ST.II/ZM, Coulter Electronics, London, UK) before and after PDGF-BB treatment for 48 h.

**Analysis of PKC activity.** Cellular PKC activity was measured using a previously published method (1). Briefly, cells in 24-well plates were stimulated with PDGF-BB for 15 min and, after the medium was removed, incubated at 37°C for 10 min with 120 μl/well of assay buffer containing, in mmol/l: 137 NaCl, 5.4 KCl, 0.3 Na₂HPO₄, 0.4 KH₂PO₄, 20 HEPES, 10 MgCl₂, 5 EGTA, 25 β-glycerophosphate, and 2.5 CaCl₂ and 1 g/l glucose, pH 7.2–7.4, 50 mg/l digitonin, 0.05 mg/ml myristoylated alanine-rich PKC kinase substrate (MARCKS) peptide, and 100 μmol/l [γ-32P]ATP (added freshly before use). To terminate the reaction, 30 μl/well of 25% trichloroacetic acid were added for 5 min, and 135 μl of the cell lysate were transferred into 1.5-ml tubes containing 15 μl of 3.75% BSA (0.375 mg/ml final concentration) and incubated on ice for 30 min. After being centrifuged for 5 min at 12,000 g, 100 μl of supernatant were dotted onto Whatman P-81 cation exchange paper (3 × 3 cm). After being washed in 75 mmol/l phosphoric acid twice for 1 min, once for 5 min, and three times for 10 min, the paper was dried and put into scintillation vials containing Instagel (3 ml/vial) for counting (5 min) in a β-counter. Nonspecific background, defined as the amount of radioactivity retained in the absence of PKC substrate MARCKS peptide, was subtracted from all values. The PKC inhibitor LY-379196 was a gift from Eli Lilly (Sydney, Australia). Similar to the compound LY-333531 (5, 25), LY-379196 at concentrations of 10–30 nmol/l selectively inhibits PKC-β activity and at 600 nmol/l induces nonsensitive PKC inhibition and can thus be used for analysis of total and specific PKC activity.

**Western blotting for protein expression of PKC subtypes.** Cells in 60-mm dishes were cultured under both normal- and high-glucose conditions in the presence or absence of E2 (10 nmol/l) and LX-379196 (30 nmol/l). Cells were lysed by incubation on ice for 30 min with lysis buffer (in mmol/l: 20 Tris base, pH 7.7, 250 NaCl, 2 EDTA, 2 EGTA, 20 β-glycerophosphate, and 1 Na-ovanate and 0.5% NP-40 and 10% glycerol; 10 μl/ml leupeptin, 5 μl/ml aprotinin, 1 μmol/l pepstatin, 1 nmol/l 4-(2-aminoethyl)benzenesulfonyl fluoride, and 10 nmol/l dithiothreitol were added before use). Plasma membrane proteins were isolated by centrifugation at 14,000 rpm for 15 min, and 30 μg of protein were electrophoresed on 10% SDS-polyacrylamide gels and transferred to Hybond enhanced chemiluminescence (ECL) filters (Sigma). The filters were blocked with 5% nonfat dry milk in TBST (20 mmol/l Tris, pH 7.5, 50 mmol/l NaCl, and 0.1% Tween-20) overnight and then washed and incubated with primary antibodies against PKC-α or PKC-β₁ (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. After being washed (3 × 10 min), blots were incubated with horseradish peroxidase-conjugated secondary antibody (DAKO) for 1 h, washed (3 × 10 min), incubated for 1 min with ECL reagents (Amersham), and exposed to X-ray films. For protein loading controls, the blots were washed again and probed with an anti-human smooth α-actin antibody (DAKO) by use of the same method as described. For quantification, bands were scanned in a PowerLook Scanner.

**Statistical analysis.** All data are presented as means ± SE. Comparisons between two means were made using Student’s t-test and multiple comparisons using ANOVA. Differences of P < 0.05 were considered significant.

**RESULTS**

E2 inhibits PDGF-BB-induced VSMC proliferation by a mechanism dependent on ER and negatively regulated by high glucose concentrations. In normal-glucose medium, preincubation of VSMC with E2 (1–100 nmol/l) for 4 h resulted in a decrease of PDGF-BB-induced DNA synthesis in a dose-dependent manner (Fig. 1), with inhibition reaching ~40% of control at 10 and 100 nmol/l. In contrast, no inhibition was observed for E2 in VSMC treated with PDGF-BB in high-glucose culture cells (Fig. 1). Consistent with the effect on DNA synthesis, direct cell counting showed a significant inhibition in PDGF-BB-stimulated increase in cell number by E2 (1–10 nmol/l), an inhibition observed for cells cultured in normal- but not in high-glucose medium (Fig. 2). High glucose itself induced an increase in cell proliferation, and the [^3H]thymidine incorporation and cell number in high glucose concentrations were higher than in normal glucose concentrations (see controls in Figs. 1 and 2). Similar results were also observed in VSMC from the aorta of Wistar Kyoto rats (data not shown).

Radioactive ligand binding assay showed that these human VSMC contained estradiol-specific binding sites (ER) at a relatively high density (24,000 ± 2,000 sites/cell; dissociation constant = 0.5 nmol/l, maximum binding capacity = 41 fmol), with no change in ER density after 48-h culture in high-glucose medium (Fig. 3). In addition, the ER antagonist ICI-182780 (100 nmol/l) completely abolished the inhibitory effect of E2.
(10 nmol/l) on cell DNA synthesis and proliferation (Fig. 4), consistent with an ER-mediated effect.

**Involvement of PKC-β in the stimulatory effect of high glucose on VSMC proliferation.** Because PKC is a key regulatory element in signal transduction and PKC-β has been implicated in diabetes-associated vascular complications (6, 12), we assessed the potential role of PKC in the effect of elevated glucose on E2 signaling by determining the effects on PKC activity and protein expression and of the PKC antagonist LY-379196.

PDGF-BB (20 ng/ml) induced a 1.3- to 1.6-fold increase in total PKC activity in these VSMC; this elevation was not further enhanced by high-glucose culture. PDGF-BB-induced increase in total PKC activity was reduced by 30% by 600 nmol/l LY-379196, whereas neither 30 nmol/l LY-379196 nor E2 affected this increase in total PKC activity (Fig. 5).

Western blotting analysis (Fig. 6) showed that protein expression of PKC-β1 increased significantly (~3-fold) in high-glucose cultures of 24 h or longer, whereas PKC-α, a protein expressed at relative high levels, was not changed in high glucose. The expression of PKC-α and -β1 was not affected by either E2 or LY-379196 (data not shown).

The difference in DNA synthesis between normal and high-glucose cultures was completely abolished by LY-379196 at 30 nmol/l (Fig. 7), the concentration for selective inhibition of PKC-β, indicating a key role of the PKC-β subtype in the action of glucose on cell growth. Under high-glucose conditions, E2 (10 nmol/l) alone did not significantly affect PDGF-BB-induced DNA synthesis but did so when added together with 30 nmol/l LY-379196 (Fig. 7), indicating that the antiproliferative effect of estradiol on VSMC is restored under high glucose conditions by selective inhibition of PKC-β.
Studies were also carried out in VSMC from the second patient by direct counting of cell numbers. The inhibitory effect of E$_2$ (1–100 nmol/l) on cell proliferation was evident in VSMC in normal glucose culture in a dose-dependent manner but was minimal in high-glucose cultures, with only 11% inhibition by E$_2$ at 100 nmol/l (Fig. 8A). Selective inhibition of PKC-$\beta$ activity by LY-379196 (30 nmol/l) reversed the antiproliferative effect of E$_2$ at 10 nmol/l, and the ER antagonist ICI-182780 abolished E$_2$ effects in these VSMC (Fig. 8B). These results were consistent with those in the first patient and indicate a general phenomenon in human VSMC.

**Fig. 4.** VSMC in 24-well plates at near confluence were growth-arrested in serum-free DMEM with NG or HG for 48 h. Cells were treated with PDGF-BB (PDGF; 20 ng/ml) for 20 h (for DNA synthesis) or 48 h (for cell proliferation) in the presence of vehicles (control), E$_2$ (10 nmol/l) alone, or E$_2$ (10 nmol/l) + ICI-182780 (ICI; 100 nmol/l). Data are shown as means ± SE of 4 wells per treatment. *P < 0.05 vs. control and PDGF+E$_2$+ICI.

**Fig. 5.** Confluent VSMC in 24-well plates were cultured in NG or HG serum-free DMEM for 48 h, pretreated with E$_2$ and LY-379196 (LY) for 4 h, and treated with PDGF-BB for 15 min, and cellular total protein kinase C (PKC) activity was determined. Data are shown as means ± SE of 4 wells per treatment. *P < 0.05 vs. PDGF-BB alone.

**Fig. 6.** Confluent VSMC in 60-mm dishes were cultured in NG or HG serum-free DMEM for 0, 6, 24 and 48 h. Membrane proteins were isolated, and specific expression of PKC-$\alpha$ and PKC-$\beta_1$ protein was detected by Western blot. Photograph shows the protein bands on a blot, by use of smooth muscle $\alpha$-actin (SM actin) protein as loading control. Bar graph shows means ± SE of the relative protein levels from 3 similar Western blots. *P < 0.01 vs. NG at same time points.

182780 abolished E$_2$ effects in these VSMC (Fig. 8B). These results were consistent with those in the first patient and indicate a general phenomenon in human VSMC.

**Fig. 7.** VSMC in 24-well plates at near confluence were continually cultured in NG or HG serum-free DMEM for 48 h, pretreated with LY-379196 (30 nmol/l) and E$_2$ (10 nmol/l) for 4 h, and treated with PDGF-BB (20 ng/ml) for 20 h. $[^{3}H]$thymidine incorporation was measured during the last 3 h of PDGF-BB treatment. Data are shown as means ± SE of two similar experiments with 6–8 wells per treatment. *P < 0.05 vs. control; #P < 0.05, comparisons of E$_2$+LY with E$_2$ alone and to LY alone in cells under HG conditions.
have higher proliferative responses to serum and PDGF and that E2 stimulates proliferation in these VSMC via activation of PKC (8). These studies indicate that estrogens have variable actions on VSMC, possibly dependent on the hormonal milieu of the cells, as well as their phenotype (20).

The present study shows that, in normal glucose, E2 attenuates PDGF-BB-induced proliferation in VSMC from human internal mammary artery, similar to our previous observations showing that E2 at physiological levels inhibits mechanical strain-induced mitosis in human aortic smooth muscle cells (11) and consistent with studies of cultured VSMC from different sources (14, 19, 23). A salient finding of the present study is that the antiproliferative effect of E2 was abolished under high glucose conditions via activation of PKC-β. Such an interpretation is supported by our observations that high-glucose medium increased PKC-β1 protein expression and selective inhibition of PKC-β (LY-379196 at 30 nmol/l) restored this antiproliferative effect of E2 in high-glucose conditions.

PDGF is an important factor in atherosclerosis. PDGF, mainly as PDGF-BB, is produced and secreted by vascular endothelial cells and contributes, via the PDGF-β receptor, to VSMC migration and proliferation (17). These PDGF actions on VSMC are believed to be mediated through a complex array of intracellular signaling pathways including MAP kinase, PKC, early growth response genes, and intracellular calcium (4). The antiproliferative effects of estrogens are reportedly mediated via the MAP kinase pathway (2, 14). In the present study, total PKC activity (Fig. 5) or PKC-β1 protein expression was not affected by E2, indicating that the inhibitory effect of E2 on PDGF-BB-induced proliferation is not via the PKC pathway. The increased activation of PKC by high-glucose medium counteracts the antiproliferative effect of E2 in VSMC. Our results show that the antiproliferative effects of estrogen are abolished by high-glucose medium but that this potentially beneficial effect of estrogen can be restored, even in a high-glucose milieu, by a PKC-β inhibitor.

Commonly used VSMC culture media such as DMEM and Waymouth's medium contain a high level of glucose (25 mmol/l), much higher than physiological levels (3–6 mmol/l). It has been shown, in the present study and other studies (24, 26), that high glucose itself induces VSMC growth, suggesting that glucose concentrations in culture media possibly influence the results of studies examining VSMC proliferation. The PKC pathway is regarded as a major mechanism underlying the vascular effects of hyperglycemia. Normalization of PKC by vitamin D (10) and of PKC-β by the selective inhibitor LY-333531 (1, 5) prevents the vascular damage of hyperglycemia in experimental diabetes. Direct effects of high glucose on cultured VSMC, as shown in the present study and other studies (10, 24, 26), are also mainly via PKC activation, especially the PKC-β isform, which, possibly via phospholipase D, induces vascular proliferation and hypertrophy, which in turn likely contribute to diabetic vascular complications.

DISCUSSION

In the present study, we have shown that E2 inhibits PDGF-BB-induced VSMC proliferation in a concentration-dependent manner but that this inhibition is lost in high-glucose cultures. We have further shown that high-glucose medium induces an approximately three-fold increase in PKC-β1 protein expression after 24-h culture and that selective inhibition of PKC-β activity with LY-379196 at 30 nmol/l inhibits high-glucose-induced cell proliferation and restores the antiproliferative effect of E2 in VSMC cultured in high-glucose medium.

The effects of estrogens on the vasculature include nongenomic vasodilatation, reduction of circulating lipids (9, 22), and genomic actions on vascular cells (15). Some studies show that estrogens inhibit growth factor-induced cell proliferation in cultured VSMC via ER-mediated reduction of mitogen-activated protein (MAP) kinase (14), c-myc, and other early-response genes (15). It has also been shown, however, that cultured smooth muscle cells from human uterine artery or aortic smooth muscle cells from pregnant rats
Our study provides a possible mechanism underlying the loss of gender-based protection against vascular disease in diabetic women. Vascular proliferation is a key element in diabetic macrovascular disease and is a significant determinant of morbidity and mortality in this condition (13). Estrogen does not appear to inhibit growth factor-induced proliferation in the presence of high glucose concentrations. Nevertheless, our findings suggest that the antiproliferative action of estrogen is restored by inhibition of PKC-β and may indicate a potential role for PKC-β inhibitors in diabetic macrovascular disease in women.

We are thankful to Dr. He Li for assistance in the PKC analysis, and Prof. J. Funder for critically reviewing the manuscript. The study was supported by a block grant from the National Health and Medical Research Council (NH&MRC) of Australia to the Baker Institute. K. Sudhir is funded as a Senior Research Fellow of the NH&MRC. S. Ling is funded by Dora Lush Scholarship from the NH&MRC as a Ph.D. candidate in Monash University at Melbourne.

REFERENCES