Progesterone inhibits human infragenicular arterial smooth muscle cell proliferation induced by high glucose and insulin concentrations

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Introduction: **Diabetes mellitus is a significant risk factor for atherosclerotic peripheral vascular disease. Hyperglycemia and hyperinsulinemia, as encountered in patients with type II diabetes, have been shown to stimulate vascular smooth muscle cell (VSMC) proliferation, a paramount feature in atherosclerosis. Female sex hormones, such as estrogen, have been suggested to inhibit VSMC proliferation. However, the role of progesterone, particularly in patients with diabetes mellitus, has not been examined. Therefore, we studied the effect of progesterone on VSMCs exposed to various concentrations of glucose and insulin.**

Methods: **Human infragenicular VSMCs isolated from the tibial arteries of five male patients with diabetes undergoing lower extremity amputation were used. Immunocytochemical studies with confocal microscopy were performed for progesterone receptor identification in these VSMCs. Cells were grown to subconfluence, followed by exposure to deprived media with various glucose (100 and 200 mg/dL) and insulin (no insulin and 100 ng/mL) concentrations. Cells were then additionally exposed to physiologic progesterone (10 ng/mL, progesterone group) and compared with a no-progesterone group. Cell count and methyl-^{3}H–thymidine incorporation were used to determine cellular proliferation. Cell count with hemocytometry was performed on day 6. DNA synthesis as reflected through methyl-^{3}H–thymidine incorporation was measured at 24 hours.**

Results: **Immunocytochemical studies with confocal microscopy showed cytosolic progesterone receptors. The no-progesterone group showed a significant rise in cell count ($P < .05$) at all concentrations of glucose or insulin compared with the control group containing 100 mg/dL glucose concentration. The no-progesterone group also showed a significant rise in thymidine incorporation ($P < .05$) in the 100 mg/dL glucose–100 ng/mL insulin group and the 200 mg/dL glucose–100 ng/mL insulin group compared with the 100 mg/dL glucose group. In the cell count studies, progesterone significantly inhibited cellular proliferation in several settings. All cell groups cultured with insulin or an elevated glucose concentration showed a significant ($P < .05$) antiproliferative effect when exposed to progesterone. With thymidine incorporation, progesterone showed a similar antiproliferative effect in cells stimulated with glucose or insulin.**

Conclusion: **Significant reductions in cell proliferation as determined with both cell count and thymidine incorporation suggest that progesterone is an inhibitor of VSMC proliferation induced by our in vitro models of hyperglycemia and hyperinsulinemia. Therefore, progesterone may have a protective role against the atherosclerotic changes associated with type II diabetes. (J Vasc Surg 2002;36:833-8.)**

Diabetes mellitus (DM) continues to remain a challenging medical problem in the United States. DM affects 6.6% of the adult population[1] and has been identified as an independent risk factor for atherosclerosis.[2] Impaired glucose tolerance (IGT), a prediabetic stage, is also associated with accelerated atherosclerosis.[3] Peripheral vascular disease involving the infragenicular vessels is common in DM and may result in limb-threatening ischemia. Type 2 DM is the most common form of the disease[4] and is characterized by insulin receptor resistance or dysfunction resulting in hyperinsulinemia and hyperglycemia. Vascular smooth muscle cell (VSMC) proliferation and migration is an important part of the atherosclerotic process and occurs as an early event in plaque formation.[5,6] Enhanced proliferation of VSMCs resulting from elevated glucose and insulin concentrations has been observed in the in vitro setting,[7] which may in part explain the acceleration of the atherosclerotic process in patients with type 2 DM or IGT.

In general, the incidence rate of cardiovascular complications in premenopausal women is lower than that in men,[8] but after menopause, the mortality and morbidity rates approximate those of age-matched men.[9] The cardioprotective effects of estrogen are well recognized; postmenopausal women given estrogen replacement therapy have a lower incidence rate of cardiovascular events (nonfatal myocardial infarction or fatal coronary disease) than do untreated patients.[10]

Possible mechanisms for this cardioprotective effect include alterations in lipid profiles (decreased low density lipoprotein and increased high density lipoprotein cholesterol levels),[11] improved endothelial function,[12] and reduced proliferation of VSMCs.[13] Estrogen and progesterone have been shown to inhibit the proliferation and

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Competition of interest: nil.

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migration of VSMC in cultures obtained from both male and female specimens[11,14,15]; however, the role of progesterone in the setting of diabetic vascular disease is unknown. Therefore, we examined human infragenicular VSMCs from patients with diabetes to determine whether they contained progesterone receptors and evaluated the effects of progesterone on the proliferation of infragenicular VSMCs in response to various concentrations of glucose and insulin.

MATERIALS AND METHODS

Harvesting and cell characterization

The protocol and informed consent form both had prior approval by the Institutional Review Board, and all subjects gave informed consent. Segments of human tibial arteries were obtained at the time of lower extremity amputation performed on five male patients with diabetes with end-stage peripheral vascular disease. The mean age of the patients was 70.8 years (±4.26 years, standard deviation). After excision of the vessels from the amputation specimen, the media were immediately dissected from the other vessel layers, minced, and incubated in a 0.8% collagenase and 0.05% elastase solution for 2 to 4 hours. The cells were centrifuged, rinsed, and immersed in a solution containing Medium-199 (Gibco BRL, Grand Island, NY), 10% fetal bovine serum (Gibco BRL), and 2% antibiotic-antimycotic (AA; 10,000 U/mL penicillin G sodium, 10,000 μg/mL streptomycin sulfate, and 25 μg/mL amphotericin B; Gibco BRL). Cells in solution were transferred to 75-cm^2 vented culture flasks and incubated at 37° C in a humidified incubator at 5% CO_2. When the cultures reached subconfluence, cells were trypsinized with 0.25% trypsin–ethylenediamine tetraacetic acid (Gibco BRL) and subcultured. This experiment used cells from passages 2 to 5, and cells were combined into a single mixed culture before transfer to wells at the beginning of this study.

Cells were identified as smooth muscle cells at first passage with a previously described immunohistochemistry technique.[16] Briefly, the cells were plated in culture chambers at 35,000 cells/mL, allowed to grow to subconfluence, and incubated overnight with primary antibodies directed against α-smooth muscle actin, myosin, vimentin, desmin, vitronectin, chondroitin sulfate, and fibronectin. Fluorescein-tagged secondary antibodies were added to the culture chambers, and cells were subsequently identified with fluorescent light microscopy.

Progesterone receptor detection

Confocal laser-scanning immunofluorescence microscopy was used to detect the presence of progesterone receptors in VSMCs. VSMCs were plated onto double-chambered slides (LabTek II, Nalge Nunc International, Naperville, Ill) previously coated with laminin (Sigma, St Louis, Mo). This was done at a density of 15,000 cells/mL per well. These cells were allowed 48 hours for adherence. They then were fixed with 1% formalin in phosphate buffered saline solution (PBS; Gibco BRL) followed by 1% formalin with 0.02% Triton X-100. Nonspecific binding was blocked with 10% normal goat serum in PBS. The slides then were washed with PBS and probed overnight at 4° C with rabbit polyclonal progesterone receptor antibody (sc-538, SCBT, Santa Cruz, Calif) in a 1:80 dilution. Control slides were prepared with the omission of the primary antibody. After washing with PBS, the slides then were probed with rhodamine conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, Pa). This was diluted to 1:1000 in 1.5% normal goat serum and PBS and placed in a humidified chamber for 1 hour. After washing, a coverslip was placed over the slide with a drop of fluorescent compatible mounting media (Sigma) and sealed with clear nail polish. The slides then were examined with an inverted confocal laser-scanning microscope (Nikon, Inc, Melville, NY). A Radiance 2000 laser scanning system (Bio-Rad Microscopy Div, Hertfordshire, United Kingdom) was the source for the argon/krypton laser beam. Samples were imaged with a broad bandpass emission filter with excitation at 568 nm with 60× oil immersion and 20× objectives. Digital images were analyzed with MetaMorph (Universal Imaging, West Chester, Pa), and figures were prepared with Adobe Photoshop (Adobe Systems, Mountain View, Calif).

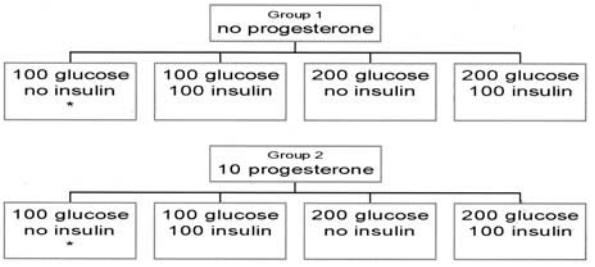

Fig 1. Flow diagram shows study design. Glucose concentration in mg/dL, insulin concentration in ng/mL, and progesterone concentration in ng/mL. *Control group.

Proliferation studies

The VSMCs were pooled into a mixed culture, grown to subconfluence, and divided into various groups to examine the effects of progesterone (Sigma) on the proliferation of VSMCs exposed to various concentrations of glucose and insulin (Fig 1). The first group, the no-progesterone group, was exposed to deprived media (M-199, 2% AA) without progesterone. This group consisted of the following glucose (Sigma) and insulin (bovine pancreas, Sigma) concentrations: 100 mg/dL glucose (control), 100 mg/dL glucose–100 ng/mL insulin (100glu-100ins), 200 mg/dL glucose (200glu), and 200 mg/dL glucose–100 ng/mL insulin (200glu-100ins). The second group of cells, the progesterone group, was likewise cultured in the same glucose and insulin concentrations with the addition of 10 ng/mL progesterone. Arterial smooth muscle proliferation was assessed with two different methods: cell count and methyl-^{3}H–thymidine incorporation.

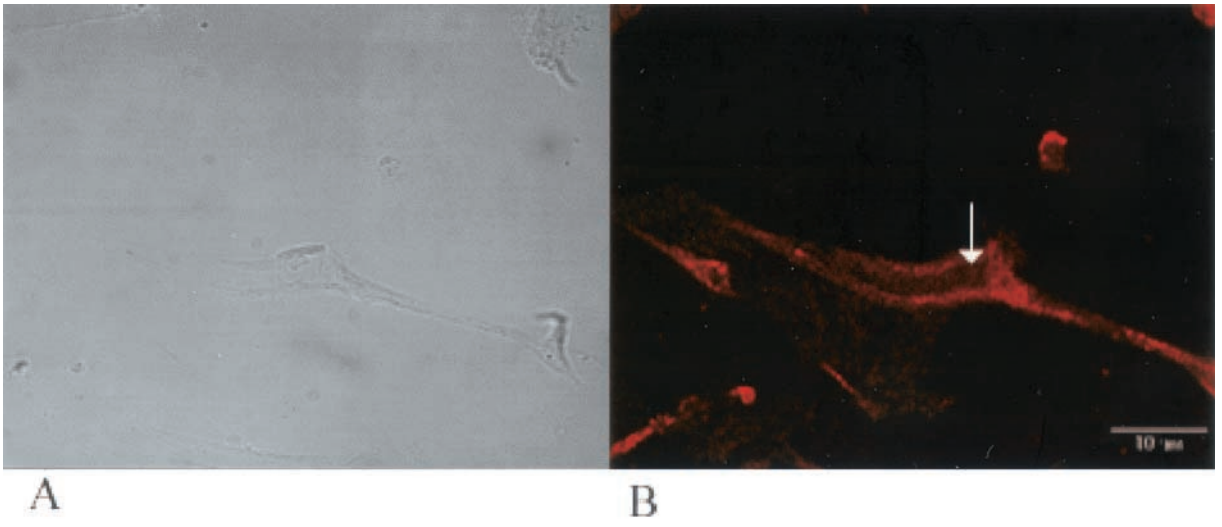

Fig 2. A, 60× Oil immersion objective transmission image of VSMC. **B,** 60× Oil immersion objective with confocal laser-scanning immunofluorescence microscopy. Cells fixed and incubated as described in Methods at dilution of 1:80 progesterone receptor antibody. Anti–progesterone receptor antibody was detected with rhodamine conjugated goat anti-rabbit secondary antibody diluted 1:1000, causing progesterone receptor to appear red. *White arrow* points to position of nucleus.

Cell count. Cells were seeded in six-well plates at 10,000 cells/mL in 2 mL of fortified media (M-199, 10% fetal bovine serum, 2% AA) and allowed 48 hours for attachment. They were subsequently incubated in deprived media for an additional 48 hours to achieve quiescence. The various groups of VSMCs were exposed to the different concentrations of glucose, insulin, and progesterone described (Fig 1). Cells were counted in triplicate with standard hemocytometry on day 6.[6] Culture medium, including glucose, insulin, and progesterone, was replaced on days 2 and 4. Values were expressed as the mean ± the standard error of the mean from six wells from each group (total of 18 experiments per group).

Thymidine incorporation. VSMCs were placed in 24-well plates at 30,000 cells/well in 1 mL of fortified medium and allowed to grow to subconfluence. Cells were exposed to deprived medium for an additional 48 hours to make them quiescent. The various concentrations of glucose, insulin, and progesterone were added as described (Fig 1). VSMCs were incubated at 37° C in these conditions for 20 hours, at which point methyl-^{3}H–thymidine (2 μCi/well; Amersham Pharmacia Biotech, Piscataway, NJ) was added. At 24 hours, VSMCs were rinsed three times with PBS. The incorporated methyl-^{3}H–thymidine was extracted with 0.2N NaOH and precipitated with 10% trichloroacetic acid before quantification in a Beckman LS6500 liquid scintillation counter (Beckman, Fullerton, Calif). Values were measured as decays per minute (DPM) and then expressed as the mean percentage ± the standard error of the mean as compared with controls from 12 wells.

Statistical analysis

Statistical analysis was performed with Sigmastat software (Jandel Scientific Software, San Rafael, Calif). One-way analysis of variance was used to compare the results of the cell count and thymidine incorporation of various glucose, insulin, and progesterone groups. Statistical significance was identified as P less than .05.

RESULTS

Cell characterization. VSMCs showed characteristic cytoskeletal proteins by exhibiting strong fluorescence of secondary antibodies when primary antibodies against α-smooth muscle actin, vimentin, fibronectin, and chondroitin sulfate were present. A weak fluorescence pattern resulted when primary antibodies against desmin and vitronectin were present. Cells incubated in the absence of primary antibodies showed no specific fluorescence.

Progesterone receptor detection. When subjected to confocal microscopy after labeling with a fluorescent antibody conjugated to a rabbit polyclonal antibody to the progesterone receptor, human VSMCs show progesterone receptor binding in red to be present in the cytoplasm of the male arterial smooth muscle cell (Fig 2, *B*). The control slides, exposed to no primary antibody, showed no fluorescence (image not shown). With the transmission image (Fig 2, *A*) viewed, the nucleus was observed and no fluorescence was present in its location (Fig 2, *B*).

Effects of glucose, insulin, and progesterone on cell proliferation. In the no-progesterone group, the addition of glucose and insulin to VSMCs synergistically increased cell proliferation as compared with control when counted

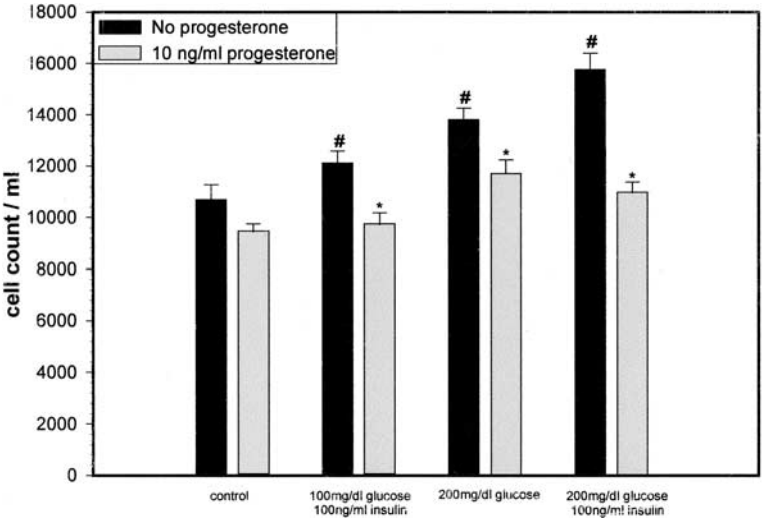

Fig 3. Effects of progesterone on smooth muscle cell proliferation determined with hemocytometer cell count in cells stimulated with various glucose/insulin combinations. $^{*}P < .05$ compared with identical glucose/insulin concentration. $^{\#}P < .05$ compared with control group.

on day 6 (Fig 3). These increases ranged from 12%, when the 100glu-100ins group was compared with the control group (12,109 ± 480 versus 10,694 ± 574; $P < .05$), to 47%, when the 200glu-100ins group was compared with the control group (15,764 ± 632 versus 10,694 ± 574; $P < .05$). When progesterone (10 ng/mL) was added, an antiproliferative effect was observed (Fig 3). An inhibition in cell proliferation occurred with significant decreases in cell count observed in the following groups: 100glu-100ins, 200glu, and 200glu-100ins. These decreases ranged from 16% (13,819 ± 445 versus 9722 ± 455; $P < .05$) in the 100glu-100ins group when progesterone was added to 30% (15,764 ± 632 versus 10,972 ± 405; $P < .05$) in the 200glu-100ins group when progesterone was added.

Effects of glucose, insulin, and progesterone on thymidine incorporation. Because of the wide variability in methyl-^{3}H–thymidine uptake depending on the degree of cell density, groups were compared with the DPM values obtained in the control group without progesterone and expressed as percent change compared with control. Control DPMs were considered as 100. In the no-progesterone group, VSMCs exposed to 100glu-100ins and VSMCs exposed to 200glu-100ins showed a significant ($P < .05$) rise in thymidine incorporation when compared with control. A significant decrease in thymidine incorporation was shown in several groups when progesterone was added (Fig 4). This effect was maximal in the group incubated with 100glu-100ins with a 58% reduction in proliferation (144% ± 5% versus 84% ± 4%; $P < .05$).

DISCUSSION

Estrogen and progesterone act through interaction with cytosolic receptors with translocation of these ligand-receptor complexes into the nucleus, where highly specific binding occurs with promotor regions of specific genes, thus altering gene expression.[13-15,17-19] Estrogen has been postulated to regulate protooncogene expression and other steroid-responsive genes, such as *c-fos* and *c-myc*.[14,19] This finding suggests that these hormones may directly affect arterial smooth muscle cell function in addition to any indirect action that may also occur, such as endothelial release of nitric oxide.

Several investigators have shown inhibition of VSMC proliferation with estrogen in various physiologic and replacement concentrations.[11,13,14,20,21] These studies used cells from both human and animal coronary arteries and saphenous veins. Estrogen suppressed proliferation and migration in VSMCs taken from these tissues. However, the role of progesterone is unclear. Moreover, no previous studies have assessed the effect of progesterone on the proliferation of VSMCs from tibial arteries exposed to increased concentrations of glucose and insulin.

We found progesterone to be a consistent inhibitor of VSMC proliferation induced by elevated glucose and insulin concentrations. Therefore, the conclusion that a progesterone-mediated antiproliferative effect in cells stimulated by elevated glucose and insulin levels may protect against the accelerated atherosclerotic complications that occur in patients with DM or IGT is reasonable.

Several possible mechanisms may explain the altered proliferation of VSMCs exposed to female hormones. First, progesterone and estrogen, through their respective receptors, may modulate gene expression at a site downstream to the proliferative cascade initiated by glucose and insulin.[19] Second, sex hormones may alter growth factor–stimulated pathways. Sahlin, Norstedt, and Eriksson[22] showed that sex steroids directly regulate the insulin-like growth factor I

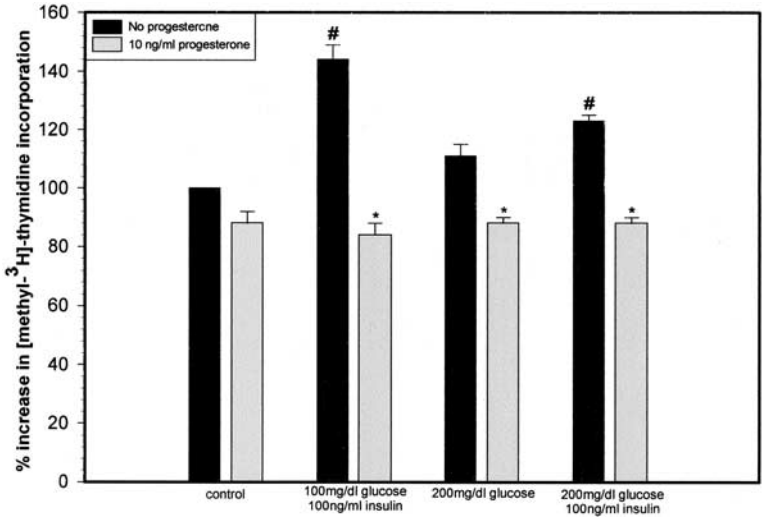

Fig 4. Effects of progesterone on cell growth determined with thymidine incorporation in cells stimulated with various glucose/insulin combinations. $^{*}P < .05$ compared with identical glucose/insulin concentration. $^{\#}P < .05$ compared with control group.

receptor. We have identified the insulin-like growth factor I receptor as the mediator of insulin's mitogenic effects in these cells,[23] so this relationship between estrogen, progesterone, and hyperinsulinemia may explain the antiproliferative effects of these sex hormones. Third, diabetic or glucose intolerant conditions may alter steroid receptor quantity and activity.[24]

Few studies have documented the effects of progesterone on VSMCs. Lee and colleagues[15] observed inhibition of VSMC proliferation and thymidine incorporation with physiologic and pharmacologic concentrations of progesterone in aortic cells from male humans. Decreases in cyclin A and E messenger RNA were also documented, which suggests interruption of the cell cycle at the G_1/S transition. The identification of progesterone receptors in the VSMCs from infragenicular origin as shown by our studies implies a specific effect of the hormone on these cells. Epidemiologic data suggest the relative risk of significant coronary artery disease is lower in women administered both estrogens and progestins as compared with those prescribed estrogens alone.[25] Our data further support that progesterone may have an important role in this risk reduction, particularly in the diabetic setting where the risk of atherosclerosis and its complications is higher.

In summary, we have shown a consistent antiproliferative effect of progesterone on VSMCs incubated in all examined glucose and insulin levels. These data suggest that progesterone possesses protective characteristics against the accelerated atherosclerotic changes associated with DM and IGT.

REFERENCES

1. Bennett PH. Epidemiology of diabetes mellitus. In: Rifkin H, Porte D, editors. Diabetes mellitus: theory and practice. New Hyde Park, NY: Medical Examination Publishing Corp (Excerpta Medica Co); 1990. p. 363-7.
2. Garcia MJ, McNamara PM, Gordon T, Kannel WB. Morbidity and mortality in diabetics in the Framingham population. Sixteen year follow-up study. Diabetes 1974;23:105-11.
3. Caballero AE, Arora S, Saouaf R, Lim SC, Smakowski P, Park JY, et al. Microvascular and macrovascular reactivity is reduced in subjects at risk for type 2 diabetes. Diabetes 1999;48:1856-62.
4. Fajans SS. Classification and diagnosis of diabetes. In: Rifkin H, Porte D, editors. Diabetes mellitus: theory and practice. New Hyde Park NY: Medical Examination Publishing Corp (Excerpta Medica Co); 1990. p. 346-56.
5. Sasaguri Y, Murahashi N, Sugama K, Kato S, Hiraoka K, Satoh T, et al. Development related changes in matrix metalloproteinase expression in human aortic smooth muscle cells. Lab Clin Invest 1994;71:261-9.
6. Ross R, Masuda J, Raines EW. Cellular interactions, growth factors, and smooth muscle proliferation in atherosclerosis. Ann N Y Acad Sci 1995;598:102-12.
7. Avena R, Mitchell ME, Neville RF, Sidawy AN. The additive effects of glucose and insulin on the proliferation of infragenicular vascular smooth muscle cells. J Vasc Surg 1998;28:1033-9.
8. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990's. Nature 1993;362:801-9.
9. Furman RH. Are gonadal hormones (estrogens and androgens) of significance in the development of ischemic heart disease? Ann N Y Sci 1968;149:822-33.
10. Stampfer MJ, Willet WC, Colditz GA, Rosner B, Speizer FE, Hennekens CH. A prospective study of postmenopausal estrogen therapy and coronary artery disease. N Engl J Med 1985;313:1044-9.
11. Moraghan T, Antoniucci DM, Grenert JP, Johnson C, Miller VM, Fitzpatrick LA. Differential response in cell proliferation to beta estradiol in coronary artery vascular smooth muscle cells obtained from mature female versus male animals. Endocrinology 1996;137:5174-7.
12. Arora S, Veves A, Caballero AE, Smakowski P, LoGerfo FW. Estrogen improves endothelial function. J Vasc Surg 1998;27:1141-7.
13. Bhalla RC, Toth KF, Bhatty RA, Thompson LP, Sharma RV. Estrogen reduces proliferation and agonist-induced calcium increase in coronary artery smooth muscle cells. Am J Physiol 1997;272:H1996-2003.
14. Dai-Do D, Espinosa E, Liu G, Rabelink TJ, Julmy F, Yang Z, et al. 17 beta-estradiol inhibits proliferation and migration of human vascular

smooth muscle cells: similar effects in cells from postmenopausal females and in males. Cardiovasc Res 1996;32:980-5.
15. Lee WS, Harder JA, Yoshizumi M, Lee ME, Haber E. Progesterone inhibits arterial smooth muscle cell proliferation. Nat Med 1997;3:1005-8.
16. Jones BA, Ali MA, Forsyth EA, Sidawy AN. Phenotypic characterization of human smooth muscle cells derived from atherosclerotic tibial and peroneal arteries. J Vasc Surg 1996;24:883-91.
17. Bayard F, Clamens S, Meggetto F, Blaes N, Delsol G, Faye JC. Estrogen synthesis, estrogen metabolism, and functional estrogen receptors in rat arterial smooth muscle cells in culture. Endocrinology 1995;136:1523-9.
18. Perrot-Applanat M. Estrogen receptors in the cardiovascular system. Steroids 1996;61:212-5.
19. Karas RH, Patterson BL, Mendelsohn ME. Human vascular smooth muscle cells contain functional estrogen receptor. Circulation 1994;89:1943-50.
20. Orimo A, Inoue S, Ikegami A, Hosoi T, Akishita M, Ouchi Y, et al. Vascular smooth muscle cells as target for estrogen. Biochem Biophys Res Commun 1993;195:730-6.
21. Fischer-Dzoga K, Wissler RW, Vesseling D. The effect estradiol on the proliferation of rabbit medial tissue culture cells induced by hyperlipemic serum. Exp Mol Pathol 1993;39:355-63.
22. Sahlin L, Norstedt G, Eriksson H. Estrogen regulation of the estrogen receptor and insulin-like growth factor I receptor in the rat uterus: a potential coupling between the effects of estrogen and IGF-1. Steroids 1994;59:421-30.
23. Avena R, Mitchell M, Carmody B, Arora S, Sidawy AN. Insulin-like growth factor 1 mediates VMSC proliferation in response to glucose and insulin. Am J Surg 1999;178:156-61.
24. Chaudhuri PK, Chaudhuri B, Patel N. Modulation of estrogen receptor by insulin and its biologic significance. Arch Surg 1986;121:1322-5.
25. Grodstein F, Stampfer MJ, Manson JE, Colditz GA, Willett WC, Rosner B, et al. Postmenopausal estrogen and progestin use and the risk of cardiovascular disease. N Engl J Med 1996;335:453-61.

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