17β-Estradiol Inhibits Flow- and Acute Hypoxia-Induced Prostacyclin Release From Perfused Endocardial Endothelial Cells

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**Background.** Because of the marked difference in the incidence and severity of cardiovascular diseases between men and premenopausal women, several groups have studied the effect of sex steroids, particularly estrogen, on vascular endothelial prostacyclin (PGI₂) release. No previous studies have addressed the effect of estrogen on endocardial endothelial cells (EECs), which are involved in the modulation of the myocardium and potentially in downstream pulmonary and systemic vascular tone. Furthermore, all previous studies of estrogen effects on cultured endothelial cell function have used cells grown under standard static cell culture conditions, thereby ignoring the contribution of flow, the ubiquitous environmental endothelial stimulus.

**Methods and Results.** The effect of 17β-estradiol pretreatment (100 ng/mL, 72 hours) on cultured sheep EEC PGI₂ release in response to multiple physiologically relevant stimuli was studied. EECs were grown in six-well plates (static conditions) or on microcarrier beads and perfused at a constant flow with normoxic (PO₂=150 mm Hg, PCO₂=35 mm Hg) or hypoxic (PO₂=35 mm Hg, PCO₂=35 mm Hg) Krebs solution. The stable metabolite of PGI₂, 6-keto-PGF₁α, was determined in samples from both static and perfusion experiments by direct radioimmunoassay. 17β-Estradiol pretreatment did not alter basal or stimulated PGF₁α release.

The vascular endothelium profoundly affects vascular smooth muscle function. Analogously, it has recently been demonstrated that endocardial endothelial cells (EECs) modulate cardiac performance. Like vascular endothelial cells, EECs possess the ability to synthesize prostacyclin (PGI₂), endothelin, and nitric oxide. Thus, the potential exists for EECs to modulate not only myocyte function but also local myocardial blood flow (paracrine effects) and possibly downstream pulmonary and systemic vascular tone (endocrine effects).

Several common cardiovascular diseases in which the endothelium is probably involved demonstrate gender differences in incidence and severity. For example, although women are more prone to primary pulmonary hypertension, the risk of cardiovascular disease at any age is lower in women than in men. This difference diminishes with advancing age and may be linked to the hormonal changes at menopause. High doses of estrogens (eg, in pregnant women and oral contraceptive users) increase the incidence of thromboembolic events in women. Gender differences in endothelial cell function have been previously suggested. Seiilan et al provided evidence of a differential effect of 17β-estradiol and testosterone on prostaglandin production by cultures of aortic endothelial cells according to the gender of the donor animal.

A clear understanding of the effects of estrogens on endothelial cell function has not emerged. Studies using a variety of in vivo and cultured cell models have shown that estrogens increase, decrease, or have no effect on PGI₂ release. Of note, previous investigators examining gender-related alterations in cultured endothelial cell function have used static endothelial cell cultures, thereby disregarding the contribution of hemodynamic forces. In vivo, endothelial cells, especially EECs, are constantly exposed to flowing blood and therefore to shear stress, which influences both the physical and biochemical characteristics of endothelial cells.

The aim of the present study was to determine whether 17β-estradiol alters EEC PGI₂ release in response to multiple physiologically relevant stimuli. The effect of 17β-estradiol on arachidonic acid-, bradykinin-, and calcium ionophore A23187-stimulated PGI₂ release was determined in samples from both static and perfusion experiments by direct radioimmunoassay.
release was examined. Because in vivo the ubiquitous environmental endothelial stimulus is flow, we studied the effect of 17β-estradiol on flow-induced EEC PG1 release. Because there are differences in the in vivo EEC oxygen environment between the right and left ventricles and because acute hypoxia is a physiologically relevant stimulus of endothelial PG1 release,16 we studied the effect of 17β-estradiol on hypoxia-induced PG1 release.

Methods

Materials

[HI]PGF1α (200 mCi/mmol) was purchased from Du Pont Co. 17β-Estradiol, arachidonic acid, A23187, and bradykinin were obtained from Sigma Chemical Co. 6-Keto-PGF1α standards and 6-keto-PGF1α antiserum were obtained from Advanced Magnetics Inc. Nu serum IV was obtained from Collaborative Research Inc. L-Glutamine, penicillin/streptomycin solution, and fungizone were obtained from Gibco. All other chemicals and reagents were from Sigma Chemical Co.

Cell Culture

EECs were cultured from postpubertal, premenopausal adult sheep, as previously described.2 Briefly, atria, valve tissue, and chordae tendineae were removed from freshly isolated female sheep hearts. The ventricles were filled with 0.1% collagenase (type IA) in Dulbecco's phosphate-buffered saline (DPBS) and incubated at 37°C for 45 minutes. The inside of each ventricle was gently rubbed with a cell scraper to remove loosely attached cells. The cell suspension was removed, pelleted by low-speed centrifugation, and resuspended in complete medium. Cells were plated, and isolated colonies of endothelial morphology were cloned using cloning rings (Belloco) and cultured in M199 supplemented with 10% FBS, 10% Nu serum IV, 50 U/mL of penicillin, 50 μg/mL of streptomyacin, 2.5 μg/mL fungizone, and 2 mmol/L glutamine and maintained at 37°C in a humidified 5% CO2 atmosphere. Cells were passaged using 0.05% trypsin/EDTA and were characterized by morphology, the presence of factor VIII, acetylated LDL uptake (Molecular Probes), and the absence of cytokeratin (AE1-AE3, Signet). Cells of passage 5 to 9 were used in experiments.

Static Culture Experiments

For static experiments, EECs were plated in six-well plates (seeding density, 100,000 cells per well) and grown to confluence in the absence or presence of 100 ng/mL 17β-estradiol (stock dissolved in ethanol and diluted with medium to working concentration) for 72 hours. Control cells received the equivalent concentration of vehicle (ethanol) only. Medium was replaced daily to maintain a stable 17β-estradiol concentration. After pretreatment, cell monolayers were washed three times with assay buffer (Hanks' balanced salt solution [HBSS] containing 1.3 mmol/L calcium and 25 mmol/L HEPES). Cells were then either incubated with or without arachidonic acid (AA, 1 μmol/L) for 2, 5, 30, or 60 minutes (to establish a time course of PG1 production) or with or without AA (1, 10 μmol/L), calcium ionophore A23187 (10 μmol/L), or bradykinin (1 μmol/L) at 37°C for 1 hour. At the appropriate times, aliquots of overlay media were collected and stored at −70°C.

Perfusion Experiments

For perfusion experiments, EECs were seeded onto 2×108 microcarrier beads (Cytosorb-3, Sigma Chemical Co) hydrated in DPBS, as detailed previously,16 and placed in 100-mL siliconized roller bottles. The roller bottles were gassed with 95% air−5% CO2, sealed, and then continuously rotated at 1.5 rpm at 37°C. When cells were confluent on the microcarrier beads, as determined by phase contrast microscopy, the medium was changed and additional microcarrier beads were added to the roller bottle (every 2 to 3 days). To determine the effect of 17β-estradiol pretreatment on flow-induced PG1 release, EECs on microcarrier beads were grown in the absence or presence of 100 ng/mL 17β-estradiol for 72 hours. After this treatment, the endothelialized microcarriers were washed three times with Krebs bicarbonate solution (pH 7.4) before being placed in cell cartridges (Gelman Sciences) for incorporation into the perfusion circuit, the characteristics of which have been previously described.16 The calculated shear stress in these cartridges ranges between 7 and 12 dyn/cm2. The cartridges containing the EECs on beads were perfused at a constant flow rate (3 mL/min) with Krebs (37°C), which had been equilibrated with either a normoxic gas mixture (21% O2, 5% CO2, 74% N2 [P02=150 mm Hg, PC02=35 mm Hg]) or a hypoxic gas mixture (4% O2, 5% CO2, 91% N2 [P02=35 mm Hg, PC02=35 mm Hg]). The effect of acute 17β-estradiol exposure was also determined. Control (untreated) EECs on microcarriers were perfused with Krebs with or without 100 ng/mL 17β-estradiol. Aliquots of effluent collected over a 2-minute period every 20 minutes were extracted using C18 Sep-Pak cartridges (Waters Associates). The lipids were eluted with methanol (extraction efficiency >95%), which was then evaporated by speedvac centrifugation. The samples were stored at −70°C until assays were performed.

Radioimmunoassay

The stable metabolite of PG1, 6-keto-PGF1α, was determined in samples from both static and perfusion experiments by direct radioimmunoassay, using standard techniques.17 Antisera used for 6-keto-PGF1α had a cross-reactivity of <0.1% for other common prostaglandins. At the end of the experimental period, cells were suspended in trypsin/EDTA for cell counting and viability measurements using the trypan blue exclusion assay or dissolved in NaOH for protein determination by the Bradford method.18 The amount of eicosanoid was indexed to the amount of cellular protein from each six-well or cell cartridge. Because timed aliquots were collected, PG1 release could be quantified per minute. Values are expressed as picograms of 6-keto-PGF1α per milligram of protein per minute (pg 6-keto-PGF1α/mg per minute).

Statistics

Data are presented as mean±SEM. ANOVA (Crunch Software Corp) was performed to compare PG1 production during static and perfusion experiments for the effect of stimulus, time, and 17β-estradiol pretreatment. During perfusion, the release of PG1 during the maximum response period (ie, 60, 80, and 100 minutes) was measured and compared for effects of 17β-estradiol and oxygen tension. Where appropriate, the method of Newman-Keuls was used to determine whether means were significantly different. A value of P<.05 was considered significant.

Results

Cell counts were not different between control cultures (no hormone addition) and cultures to which 17β-estradiol had been added (results not shown). In addition, there were no morphological differences between 17β-estradiol–treated and untreated EECs as determined by phase contrast microscopy. Cell viability, as assessed by trypan-blue exclusion, was >95% for all cell groups.

Effect of 17β-Estradiol on Stimulated and Unstimulated 6-Keto-PGF1α Release From EECs Grown in Static Conditions

The basal and AA-stimulated (1 μmol/L) 6-keto-PGF1α concentration time course in six-well plates with
or without 17β-estradiol is shown in Fig 1. Basal EEC 6-keto-PGF$_{1α}$ concentration was constant over time, whereas AA caused a time-related increase in 6-keto-PGF$_{1α}$ concentration (Fig 1). Pretreatment with 17β-estradiol did not alter the time-course profile (Fig 1). AA-stimulated 6-keto-PGF$_{1α}$ net release was greatest at 60 minutes. In subsequent six-well plate experiments, supernatant was sampled at this time.

EECs grown in six-well plates produced basal (unstimulated) levels of 6-keto-PGF$_{1α}$ that were unaffected by 17β-estradiol pretreatment (Fig 2). Unstimulated 6-keto-PGF$_{1α}$ production was 89±14 and 79±14 pg/mg per minute (n=12) for control and 17β-estradiol treated, respectively. AA-stimulated EEC 6-keto-PGF$_{1α}$ release in a dose-dependent fashion, causing a 5- and 12-fold increase in 6-keto-PGF$_{1α}$ release above unstimulated levels (Fig 2A). 17β-Estradiol did not alter this AA dose-dependent response. Calcium ionophore A23187 (10 μmol/L) and bradykinin (1 μmol/L) also stimulated EEC 6-keto-PGF$_{1α}$ release 3.5- and 7-fold, respectively, above unstimulated levels (Fig 2B). 17β-Estradiol pretreatment did not alter the response of EECs to these stimuli.

Effect of 17β-Estradiol on Flow- and Hypoxia-Induced Release of 6-Keto-PGF$_{1α}$

From Perfused EECs

Perfusion at a constant flow with normoxic Krebs solution increased EEC 6-keto-PGF$_{1α}$ release (P<.001) (Fig 3). The maximal response occurred at 60, 80, and 100 minutes. The level of 6-keto-PGF$_{1α}$ release at the initial sampling time (20 minutes) was similar in control and 17β-estradiol-pretreated EECs: control, 77±20 pg 6-keto-PGF$_{1α}$/mg per minute; 17β-estradiol pretreated, 96±22 pg 6-keto-PGF$_{1α}$/mg per minute (n=7). Additionally, it was comparable to the unstimulated release, 88±14 pg 6-keto-PGF$_{1α}$/mg per minute, by EECs in six-well plates. There was no change in 6-keto-PGF$_{1α}$ release over time in the perfused, 17β-estradiol-pretreated group (Fig 3). The mean rate of release during the period of maximum response (60, 80, and 100 minutes) in this series of experiments was 252±46 versus 100±26 pg 6-keto-PGF$_{1α}$/mg per minute for control versus 17β-estradiol-pretreated EECs, respectively (P<.005). Thus, 17β-estradiol pretreatment inhibited the flow-induced increase in 6-keto-PGF$_{1α}$ release from EECs. Acute 17β-estradiol treatment had no significant effect on control EEC 6-keto-PGF$_{1α}$ release. The mean release rates during maximum response were 328±72 and 397±69 pg 6-keto-PGF$_{1α}$/mg per minute for control EECs perfused with Krebs alone or with Krebs containing 17β-estradiol, respectively.

To determine whether 17β-estradiol had an effect on the acute hypoxic response of perfused EECs, cells pretreated with or without 17β-estradiol were perfused with normoxic or hypoxic Krebs. The mean release rates during maximum response were compared between normoxia and hypoxia. Hypoxia caused an increase in 6-keto-PGF$_{1α}$ release from control (untreated) EECs: normoxia, 140±65 versus hypoxia, 296±113 pg 6-keto-PGF$_{1α}$/mg per minute, P<.05 (Fig 4). 17β-Estradiol pretreatment inhibited the EEC response (the absolute
increase in release rate from the respective normoxic rate) to hypoxia (P < .05). During hypoxia, 17β-estradiol-pretreated EECs released significantly less 6-keto-PGF1α than control EECs: 159 ± 60 versus 296 ± 113 pg 6-keto-PGF1α/mg per minute (P < .05) (Fig 4).

**Discussion**

This study demonstrates for the first time a marked inhibitory effect of 17β-estradiol (the major circulating form of estrogen in females) on flow-induced PGI2 release from perfused EECs. In addition, 17β-estradiol inhibited hypoxia-induced EEC PGI2 release. These effects of 17β-estradiol on EECs, which are potentially involved in many aspects of cardiovascular function, may in part account for gender-related differences in cardiovascular function.

During static conditions, AA, calcium ionophore A23187, and bradykinin all increased EEC PGI2 release, consistent with a previous report from our laboratory.2 17β-Estradiol pretreatment did not alter EEC responses to these diverse stimuli or affect basal PGI2 release under static conditions. While there are no previous reports of the effect of 17β-estradiol on EEC PGI2 release, these data may be compared with results obtained in statically cultured vascular endothelium. Witter et al13 and Muck et al19 reported no effect of 17β-estradiol or 17α-ethinyl estradiol on PGI2 production by human umbilical cord and leg vein endothelium. Both these groups only examined estrogen effects on basal PGI2 release. In contrast, Seillan and coworkers8 established that both 17β-estradiol and testosterone enhanced the secretion of PGI2 and PGF2α in static endothelial cells from female piglet aortas. David et al12 demonstrated an inhibitory effect of estrogen on ATP-stimulated HUVEC PGI2 synthesis and release.12 These different results may be partly explained by differences in methodology, stimulus, gender of origin, site of endothelial origin, and species heterogeneity.

In our static experiments, 17β-estradiol did not affect EEC PGI2 release in response to increased substrate (AA) and elevated intracellular Ca2+ (induced by A23187). This suggests that 17β-estradiol did not inhibit the PGI2 synthetic pathway (ie, downstream of AA) per se. However, it is conceivable that any inhibitory effect of 17β-estradiol, such as that seen in the perfusion experiments, was overwhelmed by the concentrations of ionophore and AA used. A principal receptor-mediated mechanism by which endothelial cells respond to pharmacological stimuli involves the activation of a phosphatidylinositol-specific phospholipase C releasing the interacting second messengers diacylglycerol (DAG) and inositol trisphosphate (IP3).20 DAG activates membrane-associated protein kinase C, while elevated levels of IP3 cause release of Ca2+ from intracellular stores.21 Elevated intracellular Ca2+ activates phospholipase A2 and releases AA. The absence of an effect of 17β-estradiol on bradykinin- or calcium ionophore-stimulated PGI2 release in the static experiments suggests that 17β-estradiol does not inhibit these mechanisms. However, because other mechanisms may be involved in endothelial cell PGI2 release (eg, kinases), further studies are required to precisely define the site/s of 17β-estradiol action.

In vivo, EECs are continuously exposed to pulsatile blood flow. Complex hemodynamic shear stresses have an important role in both normal physiology and pathobiology of the endothelium. Endothelial morphology, protein synthesis, endothelium-derived growth factor production, ion channel function, and cyclooxygenase activity are all affected by perfusion.16,22,23 All previous studies of the effect of 17β-estradiol on cultured endothelial cell prostanooid production have studied cells grown in standard, static cell culture conditions. Therefore, perfusion-associated pressure and shear stress, ubiquitous environmental stimuli, were absent. In this study, to better simulate this aspect of the in vivo environment, we perfused EECs as detailed previously.16

In this study, EECs responded to perfusion with increased PGI2 release. This is consistent with studies in other endothelial cell types.16,23 An early endothelial response to shear stress appears to be activation of a potassium ion channel that regulates transmembrane K+ flux, resulting in cell hyperpolarization.23,24 It is not
known if the channel itself is the mechanosensor or if it is downstream of mechanoreceptor activation. In addition, inositol triphosphate (IP3) levels are enhanced by shear stress, suggesting involvement of intracellular calcium release. Therefore, shear stress–induced PG12 release may result from the direct action of the hemodynamic forces on the cell structure leading to K+ channel activation, mobilization of phosphatidyl inositol, calcium, and finally, prostaglandin release. In preliminary experiments we have found (data not shown) that glibenclamide (10 µmol/L) markedly decreased flow-induced PG12 release by EEC, consistent with a K+ channel role in the response of EC to flow. Our data demonstrate for the first time 17β-estradiol inhibition of the flow-induced increase in prostacyclin release from endothelium.

There are significant in vivo differences in the oxygen environments between the right and left ventricles. We have previously demonstrated that acute hypoxia increases PG12 release from perfused pulmonary artery endothelial cells. Similarly, in this study, control (untreated) perfused EECs responded to acute hypoxia with increased PG12 release. Thus EECs, like pulmonary artery endothelial cells, are capable of sensing and responding to alterations in oxygen tension by changes in eicosanoid metabolism. The mechanisms involved in the functional alterations of endothelial cells during hypoxia are not well understood. Hypoxia has been shown to increase membrane fluidity and to activate membrane phospholipases with the release of fatty acids. Hypoxia also has been reported to alter the distribution of endothelial cell membrane phospholipids with an increase in phosphatidylglycerol and enhanced cyclooxygenase activity. Altered K+ channel activity also may be implicated in the hypoxic response. During hypoxia, activation of ATP-sensitive K+ channels was reported in cardiac muscle, guinea pig aortic strips and coronary artery. Thus, a K+ channel may be involved in the EEC PG12 response to hypoxia as well as in the shear stress response. Whether 17β-estradiol alters the endothelial response to acute hypoxia has not been reported previously. In this study, 17β-estradiol inhibited the perfused endocardial endothelial response to acute hypoxia.

Estrogens may exert their effects on flow- and hypoxia-induced EEC PG12 release by several mechanisms. The inhibition of EEC PG12 release could be related to a classic hormone receptor–mediated mechanism. It is possible that their effect is exerted by modifying the activity or the total amount of phospholipase A2 (the enzyme considered to be rate limiting in generating free AA for prostaglandin synthesis), cyclooxygenase, and/or PG12 synthetase in the cells. The absence of an inhibitory effect of 17β-estradiol on PG12 release in response to AA, calcium ionophore, or bradykinin in our static experiments is not consistent with this supposition. Moreover, estradiol has been shown previously to stimulate rather than inhibit phospholipase A2 and cyclooxygenase activity albeit in smooth muscle and ureter tissue, respectively. Nevertheless, in the absence of comparative experiments demonstrating that the increase in intracellular calcium caused by 10 mmol/L A23187 or 1 mmol/L bradykinin is equal to that induced by flow or hypoxia, we cannot exclude the possibility that 17β-estradiol inhibited these mechanisms in perfused EECs. Glucocorticoids have been associated with decreased liberation of AA acid from membrane phospholipid stores. Perhaps this is also the case for estrogens, which also have membrane-stabilizing effects and effects on LDL uptake. Estrogens may also possess calcium channel–antagonistic properties. Stice et al reported that 17β-estradiol decreased calcium entry in uterine vascular smooth muscle cells. Downing et al observed that the inhibitory effect of the calcium entry blocker nifedipine was potentiated by 17β-estradiol in the rat uterine artery. If 17β-estradiol does have a calcium-antagonistic effect, then its inhibitory effect on flow- and hypoxia-induced EEC PG12 release could involve this mechanism. Our data demonstrated that 17β-estradiol acutely added to the perfusate did not inhibit flow-induced PG12 release. Inhibition was only seen with chronic pretreatment (72 hours) when the cells were perfused without 17β-estradiol. Thus, an acute effect of 17β-estradiol on membrane stability and calcium ion channels does not appear to be a likely mechanism of the inhibition of flow induced PG12 release. Finally, because K+ channel activation may be common to both flow- and hypoxia-induced PG12 release, the effect of 17β-estradiol on K+ channel activity deserves further attention.

In the ventricular lumen, endocardial prostanooids could play a role in the prevention of platelet aggregation and the occurrence of mural thrombi. Downstream pulmonary and systemic vascular tone could also respond to EEC-derived PG12. Thus, any agent influencing EEC PG12 release could have physiologically important effects in both the pulmonary and the systemic circulation. Our study indicates an inhibitory effect of 17β-estradiol on flow-and hypoxia-induced EEC PG12 release but not on pharmacologically stimulated PG12 release in static cultures. Whether the effects of 17β-estradiol reported here are related to the fact that women are more susceptible to developing pulmonary hypertension or to the suggestion that hyperestrogenemia is a factor in the development of myocardial infarction deserves further investigation. If these results in EEC can be generalized to vascular endothelial cells, then 17β-estradiol inhibition of flow- and hypoxia-stimulated PG12 release could be important in the venous system. In situations where the physiological stimulus to PG12 production is low (ie, low venous flow), hypoxia (venous O2 tension) may stimulate and thus maintain PG12 release. In this setting, inhibition of flow-generated PG12 production and inhibition of hypoxia-induced PG12 production by 17β-estradiol could synergistically contribute to venous thrombosis.

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