Abstract—To determine why pregnancy protects against intimal proliferation in a mouse model of vessel injury, we administered chorionic gonadotropin to intact and ovariectomized female mice. Chorionic gonadotropin markedly suppressed intimal proliferation in intact but not in ovariectomized female mice, indicating that the protective effects of chorionic gonadotropin require ovarian function. To test whether estrogen or progesterone might mediate the protective effects of pregnancy and chorionic gonadotropin, we administered estrogen and progesterone to ovariectomized mice. Estrogen administration to ovariectomized mice to achieve the elevated levels seen in pregnancy was sufficient to reproduce the marked suppression of intimal proliferation in response to vessel injury. Progesterone administration reduced intimal proliferation to a lesser degree and was correlated with increases in estrogen to levels seen in nonpregnant female mice. Staining for proliferating cell nuclear antigen suggested that estrogen reduced medial and intimal cell proliferation. Both the classic estrogen receptor-\(\alpha\) and the recently discovered estrogen receptor-\(\beta\) are present in vascular tissue as assessed by immunohistochemistry, providing a possible mechanism for the effects of estrogen. These results suggest that the protective effects of estrogen do not plateau at levels seen in normal females but increase further with estrogen levels up through levels seen during pregnancy. (Arterioscler Thromb Vasc Biol. 1999;19:2059-2065.)

Key Words: vascular endothelium ■ atherosclerosis ■ estrogen ■ sex ■ pregnancy

There is a well-established sex difference in the incidence of atherosclerotic disease between men and premenopausal women.\(^1\) Much of the protective effect of female sex is mediated by estrogen.\(^2,3\) In experimental models of atherogenesis and vessel injury, estrogen reduces neointimal proliferation and lesion formation.\(^4-7\) Pregnancy represents another unique hormonal state, distinct from that in males and nonpregnant females. During pregnancy, the placenta produces chorionic gonadotropin and lactogen, and maternal production of estrogens and progesterone increases dramatically. To date, the effects of these hormonal changes on vascular intimal proliferation have not been well studied.

In prior studies, we applied a cuff model of vessel injury to wild-type and endothelial nitric oxide synthase (eNOS)–mutant mice to study the role of eNOS in the neointimal proliferation response.\(^8\) This model shows the expected sex difference between male and female mice, with more intimal proliferation observed in males than in females. We found unexpectedly that pregnant mice develop almost no intima in response to vessel injury. To study further the effect of sex and pregnancy on the vascular response to injury, we ovariectomized several mice and implanted subcutaneous pumps for infusion of hormones. Ovariectomized mice develop more neointima in response to cuff placement than do intact (nonovariectomized) female mice.

To test the possible role of circulating chorionic gonadotropin in suppressing the neointimal response during pregnancy, we administered human chorionic gonadotropin (hCG) to intact female and ovariectomized mice, hCG reduces intimal proliferation in intact female mice but not in ovariectomized mice. These results indicate that hCG requires intact ovarian function for its protective effects and does not directly suppress vascular smooth muscle proliferation. Estrogen replacement to levels seen in pregnancy and with hCG administration suppresses neointimal formation. Thus, elevated estrogen levels are sufficient to mediate the protection observed during pregnancy and with hCG treatment.

Methods

Animals
Eight-week-old C57BL/6 mice (Jackson Laboratories, Bar Harbor, Me) were used for cuff placement. Some female mice underwent bilateral ovariectomy at 6 weeks of age, 2 weeks before cuff placement. All procedures were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Vascular Cuff Injury Model
The cuff injury model was performed as described previously.\(^8\) Mice were anesthetized with sodium pentobarbital (50 mg/kg), and the

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femoral arteries were exposed and isolated through a groin incision. A cuff 2.0 mm long, made from longitudinally split polyethylene-50 tubing (Clayton), was placed loosely around the left femoral artery and tied into place. The right femoral artery was dissected and isolated, but no cuff was placed (sham operation). The incisions were then closed with 4-0 sutures.

**Experimental Protocols**

Mice were divided into 7 groups: (1) male mice, (2) intact (non-variectomized) female mice, (3) ovariec-tomized mice receiving 17β-estradiol (0.1 mg/d), (5) ovariec-tomized mice receiving 17β-estradiol (0.1 mg/d) and progesterone (0.25 mg/d), (6) ovariec-tomized mice receiving hCG (8 ng/d), and (7) intact, non-variectomized mice receiving hCG (8 ng/d). Each group consisted of 5 or 6 animals. 17β-Estradiol, progesterone, and hCG were obtained from Sigma Chemical Co and were delivered by a model 2002 mini-osmotic pump (Alzet) implanted subcutaneously between the scapulas on the same day as cuff placement. The hormones were infused at a rate of 1.3 μL/h during the 14-day period between cuff placement and tissue harvesting.

**Tissue Harvesting and Histology**

Fourteen days after cuff placement, the mice were anesthetized and euthanized (100 mg/kg pentobarbital IP). A 22-gauge butterfly angiocatheter was placed in the left cardiac ventricle to allow in situ constant-pressure fixation at 100 mm Hg with 10% buffered formalin. The cuffed region and the contralateral control region of the femoral arteries were harvested, embedded in paraffin, and cut into continuous cross sections (10 μm). Parallel sections were subjected to hematoxylin and eosin staining, elastin staining, trichrome staining, and immunohistochemistry studies.

**Serum Estradiol, Progesterone, and Cholesterol Assay**

At the time of tissue harvesting, blood samples were obtained from the carotid artery. Serum estradiol and progesterone levels were measured with ELISA kits (estradiol and progesterone ELISA, Cayman Chemical Co, Inc). Serum samples were purified with a Sep-Pak C18 cartridge (Waters Co), and the assays were conducted according to the manufacturer’s instructions. Standard curves were generated using samples of known concentrations for estradiol and progesterone. The assay sensitivity was 9.0 pg/mL, and the intra-assay and interassay CVs were both 10%. Total cholesterol was measured enzymatically (Sigma), and the intra-assay and interassay CVs were 1.4% and 1.9%, respectively.

**Morphometry**

Morphometric analyses were performed on hematoxylin/eosin and elastin stained sections. Ten evenly spaced sections of each cuffed and sham-operated control artery were photographed and digitized using National Institutes of Health Image software. For each section, 4 measurements were made: luminal area, area inside the inner elastic lamina, area inside the outer elastic lamina, and vessel circumference. The intima was defined as the area between the lumen and the internal elastic lamina. The media was defined as the area between the internal and external elastic laminas. The mean vascular diameter was calculated as the vessel circumference/π. Measurements were made for all 10 sections for each vessel to obtain the mean for that vessel. Thickness and intima/media volume (I/M) ratios reported for each group reflect the average of the mean values obtained for each vessel. The measurements were done in a blinded fashion with respect to the experimental groups.

**Immunohistochemistry and Quantification of Proliferating Cell Nuclear Antigen (PCNA) Activity**

Parallel sections were used for immunohistochemistry studies. Antibodies to α-actin and CD31 were obtained from Dako Corp, antibodies to factor VIII and PCNA were from Zymed, antibodies to the progesterone receptor and estrogen receptor-β were from Affinity Biore-
Effect of Estrogen and Progesterone on Intimal Proliferation

To assess how estrogen levels are affected by pregnancy, we measured serum estrogen levels on the 7th, 14th, and 20th day of pregnancy in a separate group of pregnant C57BL/6 mice. As shown in Figure 2A, the $17\beta$-estradiol level increases during pregnancy by 3- to 4-fold over basal levels. There is an initial marked rise at 7 days, a slight decrease from the peak level at 14 days, and increases at 17 and 20 days. Thus, during pregnancy, the levels of estrogen are markedly elevated over those of nonpregnant mice.

To determine whether these elevated estrogen levels are sufficient to mediate the protection seen in pregnancy and with chorionic gonadotropin administration, we studied the effect of hormone administration on ovariectomized mice. Replacement of estrogen or progesterone by subcutaneous

Figure 1. Response to the vascular cuff injury model. The thickness of the intima (A) and I-M volume ratio (B) are shown for each group of mice: male C57BL/6 (C57M), intact female C57BL/6 (C57F), ovariectomized C57BL/6 (C57F.ov), ovariectomized C57BL/6 with estrogen (C57.ov.Er) or progesterone (C57.ov.Pr) replacement, intact female C57BL/6 treated with hCG (C57F.hCG), and ovariectomized C57BL/6 treated with hCG (C57.ov.hCG). Each group consisted of 5 or 6 mice. Ten sections were counted for each vessel, and the mean thickness and I-M volume ratios were determined for each animal. Values plotted for each group represent the mean of values obtained for each animal in the group.

Figure 2. Estrogen levels in pregnant mice and in experimental groups. A, Time course of serum estrogen levels in pregnant mice is shown for days 0 (C57F), 7, 14, 17, and 20 of pregnancy (preg). B, Serum estrogen levels are shown for each animal group. Ovariectomy (ov) reduced estradiol levels. Pregnancy, estrogen replacement in ovariectomized mice (Er), and hCG treatment of intact female mice resulted in elevations of estradiol levels to similar levels. hCG and progesterone (Pr) treatment of ovariectomized mice did not affect estradiol level. C, Serum progesterone levels are shown for each group of animals. Progesterone administration to ovariectomized animals and hCG treatment of intact animals raised progesterone levels.
osmotic pumps reduced the intimal response. The intimal thickness in ovariectomized mice that received 17β-estradiol was 2.7±0.4 μm, corresponding to an I-M ratio of 0.05 (Figure 1). Mice that received progesterone developed an intimal thickness of 16.1±2.3 μm, with an I-M ratio of 0.30. In these groups, the thickness of the media and the vessel diameter did not vary significantly, so changes in intimal thickness accounted for the changes in I-M ratio. Figure 2B shows the estrogen levels of intact, ovariectomized, estrogen-replaced, progesterone-replaced, and pregnant (day 7) mice. Ovariectomy resulted in a drop in circulating estrogen levels, from 390.6±22.8 to 193.1±53.1 pg/mL (P<0.05). Estrogen replacement in ovariectomized mice at the doses used in our study resulted in levels of estrogen comparable to those seen during pregnancy. Progesterone replacement in ovariectomized mice resulted in an increase in serum estrogen to physiological levels. Thus, estrogen replacement in ovariectomized mice to levels seen in pregnancy decreases cuff-induced intimal proliferation to the same degree as in pregnancy. hCG treatment of intact female mice also increased estrogen levels to degrees comparable with estrogen replacement and in pregnancy. hCG treatment had no effect on the estrogen levels of ovariectomized mice.

The serum progesterone level was not altered by ovariectomy or by ovariectomy plus estrogen treatment (Figure 2C). hCG treatment of intact female mice elevated the serum progesterone to levels similar to those due to progesterone treatment itself, but hCG treatment had no effect on progesterone levels in ovariectomized mice.

**Histology and Cellular Proliferation**

To characterize the histology of the intimal response, we performed a histological analysis and immunostaining of cuff-injured vessels, as shown in Figure 3. Elastin stains, which stain the elastin fibers black, and Accustain trichrome, which stains the collagen fibers green, did not reveal differences in the amount or arrangement of collagen or elastin fibers in the medial and adventitial layers among the groups. The medial and intimal cells were stained by using a monoclonal antibody directed against smooth muscle α-actin. The cells closest to the lumen were stained by using an antibody directed against factor VIII–related antigen and CD31, consistent with their being endothelial cells. There were no differences detected in the staining of endothelial cells with the use of an anti-eNOS antibody among the groups.

We examined the expression of PCNA as a marker of cell proliferation in the vessel wall. PCNA staining for each of the groups is shown in Figure 4. The mean number of PCNA-positive cells was higher in the intimas of male mice (102±3.1) after cuff injury than in female mice (59±5.2, P<0.01). Ovariectomy increased the number of PCNA-positive cells in the intima (116.1±11.9, P<0.001). Estrogen and progesterone treatments of ovariectomized mice suppressed the number of PCNA-positive cells to 14.3±1.7 (P<0.0001) and 58.5±7.8 (P<0.0003), respectively, paralleling the effects of this treatment on intimal thickness and I-M ratio. hCG treatment reduced the number of PCNA-positive intimal cells in intact females (13.8±2.8, P<0.01), but not in ovariectomized females (113.8±7.3). In most of the groups, the number of intimal PCNA-positive cells was correlated with the increase in intimal thickness. However, in ovariectomized mice and in ovariectomized mice treated with hCG, the increase in intimal thickness was more pronounced than the increase in PCNA-positive cells, suggesting that there may be an increase in the amount of tissue matrix as well.

**Expression of Estrogen and Progesterone Receptors**

Most tissues that respond to estrogen contain estrogen receptors. To determine whether cells within the vasculature express estrogen receptors that might mediate the protective effects of estrogen, we used immunohistochemistry to stain for the estrogen receptors-α and -β in the femoral artery. Staining of sections from ovary and oviduct were used as positive controls and for comparison. Estrogen receptors-α and -β were both found in femoral arteries and veins of male and female mice. The cells that stain for these receptors include endothelial cells, smooth muscle cells, and fibroblasts in the adventitia, as well as cells lining small vessels within the adventitia (Figure 6). The staining intensity for both receptors was stronger in the endothelial cells than in smooth muscle cells. Both nuclear staining and cytoplasmic staining
were observed. Progesterone receptor staining was present in the nuclei of intimal cells, medial smooth muscle cells, and adventitial cells. Negative control sections did not show nonspecific staining.

**Discussion**

Pregnancy is associated with vascular changes, including systemic vasodilation, increased cardiac output, and increased blood volume. These effects are thought to be mediated by hormonal changes. Chorionic gonadotropin, placental lactogen, and prolactin are newly synthesized, and maternal estrogen levels and progesterone levels rise. We had previously found an unexpected marked reduction in the neointimal response to cuff injury in pregnant mice, a protective effect that overrode even the effect of eNOS gene deletion. This led us to investigate whether changes in chorionic gonadotropin or ovarian hormones might mediate this protection.

Chorionic gonadotropin, made by the syncytiotrophoblast, “rescues” the corpus luteum and stimulates progesterone production. Levels of chorionic gonadotropin rise early and remain detectable throughout pregnancy. Chorionic gonadotropin is a dimer of α- and β-subunits. The α-subunit is shared with luteinizing hormone, follicle-stimulating hormone, and thyroid-stimulating hormone, whereas specificity is conferred by the β-subunit. Thus, we used the β-subunit of hCG (ie, βhCG) in our studies. We found that βhCG suppressed the intimal response of intact female mice but not of ovariectomized mice. Thus, chorionic gonadotropin does not act directly on vascular smooth muscle but rather causes its effects by modulating ovarian hormone production.

Estrogens may mediate this effect, since ovarian production of estrogen increases markedly during pregnancy. We found that estradiol levels in pregnant mice increased to 1801 pg/mL, compared with levels of 390 pg/mL in nonpregnant mice. The estrogen level of hCG-treated, intact female mice, but not of hCG-treated ovariectomized mice, rose to levels comparable to those in pregnant mice. Estrogen replacement in ovariectomized mice to achieve estradiol levels in the same range as seen in pregnancy also led to a marked reduction in neointimal proliferation. These results indicate that increases...
in estrogen levels are sufficient to account for the protection seen in pregnancy.

The mouse cuff model demonstrates a protective effect of female sex. In this study, we have shown that ovariectomy obliterates the protective effect of female sex. The I/M ratio of ovariectomized mice was twice as high as that in intact female mice and was even higher than that in normal male mice. Estrogen replacement reduced neointimal proliferation in this model, as it does in other models of vessel injury, including balloon injury models of the rabbit common iliac artery,5 the filament injury model of the rabbit common iliac artery,4 and rat carotid artery,6 and the cuff injury model of the rat carotid artery23 and an ovariectomized, cholesterol-fed model of apoE–deficient mice by 17β-estradiol is not affected by NOS inhibitors.20 A sex difference in vascular response to cuff injury persists in eNOS-mutant mice, showing that some estrogen effects do not depend on eNOS.6 In the cuff model of vessel injury, the most relevant protective effects of estrogen appear to be direct effects on vascular smooth muscle growth and proliferation. Estrogens reduce thymidine uptake in porcine coronary artery explants.21 Effects on balloon-injured vessels in mice are reduced by estrogens.1,5 In the current study, we used immunohistochemistry for PCNA as a measure of cell proliferation.22 PCNA staining of both the media and intima were dramatically reduced after estrogen treatment.

There are conflicting reports on whether progesterones, used clinically to counter the potential neoplastic effects on the endometrium of unopposed estrogens, attenuate the vasculoprotective effects of estrogen. Two studies that have shown such an effect include a balloon injury model of the rat carotid artery24 and an ovariectomized, cholesterol-fed model in rabbits.25 In contrast, another study in rabbits did not show such an effect of progesterone.25 In our study, progesterone did not appear to interfere with the vasculoprotective effect of estrogen. Progesterone levels were nearly doubled by hCG treatment of intact female mice, whereas they were not elevated at all in ovariectomized mice treated with estrogen. Despite these differences, the degree of protection against neointimal formation was equivalent in these 2 groups with supraphysiological estrogen levels. Progesterone also did not interfere with the protective effect of estrogen at physiological levels. Progesterone administration to ovariectomized mice also doubled progesterone levels, yet the degree of intimal proliferation was equivalent in this group and in intact females.

Receptors for estrogen mediate some of its biological effects.26–28 Both the classic estrogen receptor-α and the recently identified estrogen receptor-β29 have a similar high affinity for estradiol. However, some ligands show different relative affinities for the 2 receptors, and their tissue distribution and relative levels also vary. We found specific staining for both α and β in the ovaries, oviduct, brain, and blood vessels. Estrogen receptor-α and β staining was seen in intimal cells, medial smooth muscle cells, and adventitial cells. Estrogen receptor-β staining was predominantly more nuclear than cytoplasmic in the vessels. Staining in vessels was weaker than in the ovary and oviduct for both receptors (Figure 6), and progesterone receptor staining was stronger than estrogen receptor staining in vessels. Progesterone re-
ceptin staining was predominantly nuclear and appeared in the endothelium, medial smooth muscle, and adventitia. Progesterone receptors are present in human vessels, although their role in modulating the events involved in atherosclerosis are not clear.

Our results demonstrate that the protective effect of pregnancy in the cuff injury model can be replicated by administration of chorionic gonadotropin or by elevated levels of estradiol. The effect of chorionic gonadotropin depends on intact ovarian function, because hCG had no vasculoprotective effect in ovariectomized mice. hCG itself has growth-stimulatory effects, which may explain the increased intimal and medial thicknesses of the hCG-treated ovariectomized mice compared with untreated ovariectomized mice. However, the I/M volume ratios were comparable. Our study does not address the potential effects of other hormones present during pregnancy, such as placental lactogen, which has growth hormone and prolactin-like effects. The estradiol levels of pregnant female mice, hCG-replaced intact females, and estrogen-replaced ovariectomized mice were all similar and were higher than physiological levels seen in normal, intact, cycling female mice. Estrogen levels were correlated with the degree of vascular protection, suggesting that ovarian estrogen synthesis may mediate the protective effects of pregnancy and hCG treatment. Furthermore, the protective effects of estrogen did not plateau at levels seen in female mice but continued to increase with higher estrogen levels, as seen in pregnant mice, hCG-treated, intact female mice, and estrogen-treated, ovariectomized mice. With further advances in our understanding of the molecular mechanisms by which estrogen acts, it may be possible to safely and specifically suppress the vascular responses to injury beyond the degree naturally observed in nonpregnant females.

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