Determinants of Flow-Mediated Outward Remodeling in Female Rodents
Respective Roles of Age, Estrogens, and Timing

Kahena Tarhouni, Anne-Laure Guihot, Emilie Vessières, Bertrand Toutain, Vincent Procaccio, Linda Grimaud, Laurent Loufrani, Francoise Lenfant, Jean-Francois Arnal, Daniel Henrion

Objective—Flow (shear stress)-mediated outward remodeling (FMR) of resistance arteries is a key adaptive process allowing collateral growth after arterial occlusion but declining with age. 17-β-estradiol (E2) has a key role in this process through activation of estrogen receptor α (ERα). Thus, we investigated the impact of age and timing for estrogen efficacy on FMR.

Approach and Results—Female rats, 3 to 18 months old, were submitted to surgery to increase blood flow locally in 1 mesenteric artery in vivo. High-flow and normal-flow arteries were collected 2 weeks later for in vitro analysis. Diameter increased by 27% in high-flow arteries compared with normal-flow arteries in 3-month-old rats. The amplitude of remodeling declined with age (12% in 18-month-old rats) in parallel with E2 blood level and E2 substitution failed restoring remodeling in 18-month-old rats. Ovariectomy of 3-, 9-, and 12-month-old rats abolished FMR, which was restored by immediate E2 replacement. Nevertheless, this effect of E2 was absent 9 months after ovariectomy. In this latter group, ERα and endothelial nitric oxide synthase expression were reduced by half compared with age-matched rats recently ovariectomized. FMR did not occur in ERα−/− mice, whereas it was decreased by 50% in ERα+/− mice, emphasizing the importance of gene dosage in high-flow remodeling.

Conclusions—E2 deprivation, rather than age, leads to decline in FMR, which can be prevented by early exogenous E2. However, delayed E2 replacement was ineffective on FMR, underlining the importance of timing of this estrogen action. (Arterioscler Thromb Vasc Biol. 2014;34:00-00.)

Key Words: blood flow velocity • estrogens • ventricular remodeling

The nitric oxide (NO) pathway has an important protective role in heart and blood vessels, and shear stress attributable to blood flow is a potent endogenous stimulus for endothelial NO synthase (eNOS) expression level. A decreased NO production by endothelial cells is associated with higher risk of cardiovascular diseases, and a reduced responsiveness to flow (flow-mediated dilation) is the hallmark of endothelium dysfunction. Chronic increases in blood flow induce outward hypertrophic remodeling in resistance arteries associated with improved NO-dependent dilation. This remodeling occurs in physiological situations, such as growth, pregnancy, or exercising, and has a key role in postischemic revascularization because it allows collateral arteries growth and angiogenesis. Nevertheless, the ability of resistance arteries to remodel in response to a chronic increase in blood flow is reduced in rat models of aging, hypertension, and diabetes mellitus. It should be noted that these studies were performed in male rats only, and that we recently demonstrated an important role of female sex hormones in the mechanism of flow-mediated remodeling (FMR). Indeed, FMR is absent in ovariectomized female rats and in mice lacking the estrogen receptor α (ERα). Epidemiological studies have demonstrated that women, before menopause, are better protected than men against cardiovascular diseases. Indeed, vascular and heart cells contain specific high-affinity receptors for estrogen in both humans and animals. Furthermore, estrogens stimulate eNOS leading to NO production. Both animal and human studies have shown that the decline in ovarian function is associated with decreased NO production. Stimulation of the NO-pathway may explain, at least in part, the protective effect of estrogens on the vascular wall. However, some aspects of vascular protection, such as atheroprotection, are not dependent on NO, showing that multiple mechanisms concur to estrogen-dependent protection, although endothelial ERα again plays a prominent role. Nevertheless, a controversy remains regarding the beneficial effect of estrogen treatment after menopause. This beneficial effect has been questioned.
after 2 interventional studies, the Heart and Estrogen/Progestin Replacement Study (HERS) and the Women’s Health Initiative (WHI). The initial results suggested a negative impact of the treatment, leading to a worldwide reduction in the use of substitution therapies. Nevertheless, recent publications with a more detailed analysis of the data countered the initial conclusion. Interestingly, a major difference in the effect of hormones between younger and older women has been shown. Indeed, the treatment tended to confer coronary protection in women treated within 10 years after menopause, whereas a latter treatment may induce deleterious effects. The mechanisms accounting for the impact of timing in the atheroprotective action of estrogens has been recently reviewed. In particular, it has been described, in the atheroma plaque, a decreased ERα expression and a reduction in 27-hydroxycholesterol (27HC) production. Indeed, 27HC is a competitive antagonist of ER action, which can counteract the protective action of 17-β-estradiol (E2).

To better understand to which extent a timing effect also can impact the function of arterioles, we investigated FMR in 3-month-old female rats as a model of surgical menopause. A 15-year review of the Atherosclerosis Risk in Communities (ARIC) study and the Women’s Health Initiative (WHI), the Heart and Estrogen/Progestin Replacement Study (HERS) and the Women’s Health Initiative (WHI). The initial results suggested a negative impact of the treatment, leading to a worldwide reduction in the use of substitution therapies. Nevertheless, recent publications with a more detailed analysis of the data countered the initial conclusion. Interestingly, a major difference in the effect of hormones between younger and older women has been shown. Indeed, the treatment tended to confer coronary protection in women treated within 10 years after menopause, whereas a latter treatment may induce deleterious effects. The mechanisms accounting for the impact of timing in the atheroprotective action of estrogens has been recently reviewed. In particular, it has been described, in the atheroma plaque, a decreased ERα expression and a reduction in 27-hydroxycholesterol (27HC) production. Indeed, 27HC is a competitive antagonist of ER action, which can counteract the protective action of 17-β-estradiol (E2).

To better understand to which extent a timing effect also can impact the function of arterioles, we investigated FMR in 3-month-old female rats as a model of surgical menopause.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Estradiol Blood Level and FMR
Fourteen days after arterial ligation, arterial diameter was determined in vitro in response to stepwise increases in intraluminal pressure. Passive arterial diameter was significantly higher in high-flow (HF) than in normal-flow (NF) arteries in 3-month-old female rats (Figure 1A). The percentage of increase in diameter in HF arteries declined progressively with age in 3- to 18-month old female rats (Figure 1B). Interestingly, E2 blood level also decreased progressively with age (Figure 1C), although these endogenous levels were sufficient to maintain a normal uterus weight, with the exception of a slight decline in 18-month-old rats (Figure 1D).

In 18-month-old female rats treated for 3 weeks with exogenous E2, E2 blood level met those levels measured in young rats (24±4 pg/mL in E2-treated 18-month-old rats versus 20±4 pg/mL in 3-month-old rats and 4±1 pg/mL in 18-month-old rats without treatment). Nevertheless, remodeling did not differ between treated and untreated rats (Figure 1E) despite a significant effect of E2 on uterus weight (660±83 mg with treatment versus 426±69 mg without treatment).

Endogenous Estradiol and FMR
E2 blood level and uterus weight were significantly reduced in ovariectomized rats compared with that in control and E2-treated ovariectomized rats, whereas mean arterial pressure and body weight were not affected by the ovariectomy or by the treatment with E2 (Table). FMR in 3-month-old female rats did not occur in ovariectomized rats (Figure 1 in the

Figure 1. Arterial diameter measured in response to stepwise increases in pressure in mesenteric arteries submitted chronically to high flow (HF) or to normal flow (NF). Arteries were isolated from 3- and 18-month-old female rats (A and B). To facilitate intergroup comparison, the increase in diameter attributable to the chronic increase in blood flow was expressed as percentage increase in diameter (HF−NF / NF×100, B). Estradiol (E2) blood level (C) and uterus weight (D) were measured in these rats. Arterial diameter was also measured in HF and NF arteries isolated from 18-month-old intact female rats treated or not with E2 (E). Mean±SEM is represented (n=12 rats per group). *P<0.05, HF vs NF. #P<0.05 vs 3- or 6-month-old rats.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>E2</td>
<td>17-β-estradiol</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<tr>
<td>FMR</td>
<td>flow-mediated remodeling</td>
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<tr>
<td>HF</td>
<td>high flow</td>
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<tr>
<td>NF</td>
<td>normal flow</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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online-only Data Supplement). In ovariectomized rats treated with E2, remodeling was equivalent to control, confirming that E2 is involved in remodeling.

**Prolonged Ovariectomy and FMR**

In 6-month-old rats, ovariectomy prevented HF remodeling (ie, diameter under 100 mm Hg was 388±18 versus 375±21 μm in intact rats, n=8 per groups), whereas after ovariectomy followed by immediate treated with E2, HF remodeling was equivalent to that in intact rats (Figure II in the online-only Data Supplement).

In 6-month-old rats ovariectomized at 3 months (Figure IIC and IID in the online-only Data Supplement), FMR did not occur but exogenous E2 restored in part remodeling (10±3% versus 22±4% increase in diameter in HF versus NF arteries, *P*<0.001).

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**Table. Body Weight, Mean Arterial Pressure, and Uterus Weight Measured in 3- to 12-Month-Old Female Rats and Submitted to 3-Week, 3-Month, or 9-Month Ovariectomy**

<table>
<thead>
<tr>
<th>Rats</th>
<th>Control</th>
<th>Control+OVX (3 wk) +E2 (2 wk)</th>
<th>3 wk or 6–9 mo OVX</th>
<th>6–9 mo OVX+E2 (2 wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight, g</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>3-month-old rats</td>
<td>222±5</td>
<td>233±3</td>
<td>228±4</td>
<td>225±6</td>
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<tr>
<td>6-month-old rats</td>
<td>360±5</td>
<td>342±8</td>
<td>357±6</td>
<td>351±7</td>
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<tr>
<td>12-month-old rats</td>
<td>326±10</td>
<td>352±5</td>
<td>379±12</td>
<td>347±10</td>
</tr>
<tr>
<td><strong>Mean arterial pressure, mm Hg</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-month-old rats</td>
<td>95±5</td>
<td>97±5</td>
<td>90±2</td>
<td>91±4</td>
</tr>
<tr>
<td>6-month-old rats</td>
<td>95±4</td>
<td>94±2</td>
<td>101±5</td>
<td>93±4</td>
</tr>
<tr>
<td>12-month-old rats</td>
<td>97±4</td>
<td>98±3</td>
<td>97±4</td>
<td>100±3</td>
</tr>
<tr>
<td><strong>Uterus weight, g</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3-month-old rats</td>
<td>0.61±0.04</td>
<td>0.52±0.09</td>
<td>0.20±0.02*</td>
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<tr>
<td>6-month-old rats</td>
<td>0.65±0.09</td>
<td>0.716±0.03</td>
<td>0.24±0.04*</td>
<td>0.50±0.03</td>
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<tr>
<td>12-month-old rats</td>
<td>0.54±0.15</td>
<td>1.12±0.27</td>
<td>0.291±0.08*</td>
<td>0.62±0.07</td>
</tr>
</tbody>
</table>

Rats were treated or not with 17β-estradiol (E2) for 2 weeks or not. Mean±SEM is shown (*n*=12 rats per group). OVX indicates ovariectomized.

*P*<0.05 vs control.

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**Figure 2.** Arterial diameter measured in response to stepwise increases in pressure in mesenteric arteries submitted chronically to high flow (HF) or to normal flow (NF). Arteries were isolated from 12-month-old female rats. Rats were intact rats (A), rats ovariectomized (OVX) at 12 months (B) and treated with 17β-estradiol (E2), rats ovariectomized at 3 months (C) and rats ovariectomized at 3 months and treated with E2 at 12 months. Mean±SEM is represented (*n*=12 rats per group). *P*<0.05, HF vs NF arteries.
In 12-month-old rats, intact or ovariectomized and immediately treated with E2, FMR was significant (Figure 2A and 2B). No remodeling occurred in rats ovariectomized at 12 months (ie, diameter <100 mm Hg was 406±20 versus 394±19 μm in intact rats, n=8 per groups).

In 12-month-old rats ovariectomized at 3 months, FMR did not occur (Figure 2C), and a treatment with E2 was inefficient (Figure 2D).

E2 blood level and uterus weight were significantly reduced in ovariectomized rats at 3 months and in rats ovariectomized at 6 or 12 months. In all groups, the treatment with E2 increased uterus weight to control level (Table).

**Prolonged Ovariectomy and Compensatory Increase in Arterial Wall Mass**

In 12-month-old rats, intact or ovariectomized+E2, cross-section area (Figure 3A) and media thickness (Figure 3B) were higher in HF than in NF arteries. In rats ovariectomized for 9 months, treated or not with E2, cross-sectional area and media thickness in HF arteries were not significantly higher compared with NF arteries (Figure 3A and 3B). Wall-to-lumen ratio was similar in HF and NF arteries in all 4 groups (Figure 3C); nevertheless, it was significantly greater in rats ovariectomized for 9 months (with or without E2) compared with the 2 other groups.

**Endothelium (NO)-Mediated Relaxation**

In rats ovariectomized at 12 months, a treatment with E2 improved acetylcholine-mediated dilation significantly, whereas in rats ovariectomized at 3 months, E2 did not change acetylcholine-mediated dilation (Figure 4A). Acetylcholine-mediated relaxation was fully dependent on the production of NO because N(omega)-nitroarginine methyl ester suppressed relaxation in all groups (Figure 4B). Endothelium-independent relaxation induced by sodium nitroprusside was significantly higher in rats ovariectomized at 3 months compared with rats ovariectomized at 12 months, irrespective of the treatment with E2 (Figure 4C).

**ERα and eNOS Expression Level**

In 12-month-old rats ovariectomized at 12 months and treated with E2 and in intact 12-month-old rats, ERα and eNOS expression level was higher than that in 12-month-old rats ovariectomized at 3 months, treated or not with E2 (Figure 5A). Moreover, in 12-month-old rats ovariectomized at 12 months and treated with E2 and in intact 12-month-old rats, ERα and eNOS expression level was higher in HF than in NF vessels as evidenced by a ratio of HF to NF ranging from 1.3 to 1.8 (Figure 5B). This was not observed in 12-month-old rats ovariectomized at 3 months, treated or not with E2 (Figure 5B). The ratio of phosphorylated eNOS to eNOS was similar in the 4 study groups (Figure III in the online-only Data Supplement).

**ERK1/2, NADP(H) Oxidase, and SOD**

ERK1/2 expression level tended to be higher in 12-month-old rats ovariectomized for 9 months whether they were treated with E2 or not. The ratio of phosphorylated ERK1/2 to ERK1/2 was similar in the 4 study groups (Figure IV in the online-only Data Supplement).

**Figure 3.** Media cross-section area (CSA; A), media thickness (B), and wall-to-lumen ratio (C) obtained in mesenteric arteries submitted chronically to high flow (HF) or to normal flow (NF). Arteries were isolated from 12-month-old intact rats (12 months), 12-month-old rats ovariectomized (12-month OVX) at 12 months, and treated with 17α-estradiol (E2; OVX+E2 at 12 months), 12-month-old rats ovariectomized at 3 months and left untreated (9-month OVX), and 12-month-old rats ovariectomized at 3 months and treated with E2 at 12 months (9-month OVX+E2). Means±SEM is represented (n=12 rats per group). *P<0.05, HF vs NF arteries. #P<0.05, Rats with 9 months OVX compared with the corresponding 12-month-old rats.
expression levels measured in HF vessels to that measured in NF arteries was not significantly different among the 4 study groups.

**Inflammatory Response**

Tumor necrosis factor α, COX-2, and HO-1 expression levels were not significantly affected by ovariectomy with or without E2-treatment (Figure IX in the online-only Data Supplement).

**FMR in ERα Knockout Mice**

Diameter expansion occurred in wild-type (Figure 6A) and ERα−/− mice (Figure 6B) and not in ERα−/− mice (Figure 6C). However, the amplitude of remodeling (Figure 6D) in ERα−/− mice was half that observed in ERα+ mice.

**Discussion**

The main finding of the present study is that estrogens prevent the decline in FMR occurring with age, and that deprivation of E2, rather than age, leads to a rapid decline in FMR. A gap of 9 months between endogenous estrogen deprivation and exogenous E2 substitution impairs E2 action, underlining the importance of timing of E2 effect on this vascular process. A 2-fold decrease in both ERα and eNOS expression levels, associated with reduced NO-dependent relaxation, could play a key role in this process.

A chronic increase in blood flow induces changes in structure and function of arterioles or resistance arteries with an increase in diameter and wall mass (hypertrophy) and improved endothelium-dependent dilatation. This remodeling has a major role in growth, pregnancy, and exercising as well as in ischemic diseases. Indeed, it is important for collateral arteries growth after occlusion of a larger artery, and consequently it is essential for postischemic revascularization.

FMR is strongly reduced in physiological aging as evidenced in male rats 12 to 24 months old. It is also reduced or absent in diseases associated with disturbed local blood flow supply, such as obesity, diabetes mellitus, and hypertension. Nevertheless, these studies have been performed in young male rats, and limited information is available in female rats. In humans, the probability to face cardiovascular events increases after 40 years, especially in men, because women are better protected than men before menopause. In agreement with these observations, we found that remodeling was maintained in 3- to 6-month-old rats. This was correlated with a progressive decline in circulating E2 level. Nevertheless, treating 18-month-old female rats with E2 did not increase the amplitude of remodeling, suggesting that irreversible changes occurred.

To better understand this latter observation, remodeling was then investigated in young female rats submitted to prolonged estrogen deprivation to mimic menopause experimentally without interaction with the effect of aging. We evidenced a nonreversible loss of the capacity of E2 to improve remodeling after prolonged estrogen deprivation. This finding is important with regard to the controversy existing on the beneficial effect of estrogen treatment after menopause. Indeed, despite the results of the initial trials that brought out the controversy, more recent works show that estrogen substitution therapy is efficient in women treated...
within the decade after menopause.\(^1\)\(^4\) Furthermore, the present study is also of interest for the management and treatment of women with primary ovarian insufficiency.\(^3\)\(^5\)\(^6\)

Whereas E2 lost its efficacy on FMR after prolonged ovariectomy, HF arteries are able to develop hypertrophy so that the wall-to-lumen ratio was excessively high. This has been associated with the occurrence of vascular disorders, strengthening the importance of E2 and timing in the protection of the vascular wall.

We have previously shown that E2 and ER\(\alpha\) are important for the last phase of FMR, with activation of the expression of eNOS needed for the diameter expansion.\(^1\)\(^4\) The findings of the present study are in agreement with our previous observation with no obvious effect of E2 on inflammation and oxidative stress. Nevertheless, FMR of resistance arteries depends on eNOS expression level and NO production\(^7\)\(^8\) under the influence of ER\(\alpha\).\(^1\)\(^4\) The expression level of eNOS and ER\(\alpha\) and their activity are modulated by E2.\(^3\)\(^9\) Moreover, the disruptive mutation in the estrogen receptor gene described in 1 man\(^4\)\(^0\) is associated with absence of flow-mediated dilation of the brachial artery,\(^4\)\(^1\) and flow-mediated dilatation is reduced in men.

**Figure 5.** The expression level of endothelial nitric oxide synthase (eNOS, left) and the estrogen receptor \(\alpha\) (ER\(\alpha\), right) was measured using Western-blot analysis in mesenteric arteries isolated from 12-month-old rats ovariectomized at 12 months (OVX) and treated or not with 17-\(\beta\)-estradiol (E2) at 12 months (A). Rats were also 12-month-old rats ovariectomized at 3 months (9 months OVX), treated or not with E2 at 12 months. The ratio of protein expression in high-flow (HF) arteries to that measured in normal-flow (NF) vessels is shown in (B). MeansSEM is represented (n=12 rats per group). *\(P<0.05\) vs 12-month-old rats (blue).

**Figure 6.** Arterial diameter measured in response to stepwise increases in pressure in mesenteric arteries submitted chronically to high flow (HF) or to normal flow (NF). Arteries were isolated from 4- to 5-month-old female mice. Mice were estrogen receptor \(\alpha\) (ER\(\alpha\))\(^+\)\(^+\) (A), ER\(\alpha\)\(^+\)\(^−\) (B), and ER\(\alpha\)\(^−\)\(^−\) (C). The increase in diameter attributable to the chronic increase in blood flow was expressed as percentage increase in diameter (HF−NF/NF×100, D). MeansSEM is represented (n=8 rats per group). *\(P<0.05\), HF vs NF arteries (D). #\(P<0.05\), +/- vs +/-.
treated with aromatase inhibitors. These studies suggest that the acute endothelial response to flow (shear stress) depends on ERα activation. Furthermore, in premenopausal women, endothelial ERα expression level is modulated by estrogens and correlated to eNOS expression and NO-dependent dilation of the brachial artery; ERα and eNOS expression levels as well as flow-dependent dilation being reduced in menopausal women. In agreement with this study, we found that ERα and eNOS expression levels are higher in intact 12-month-old rats than in ovariectomized age-matched rats. Nevertheless, a main finding of the present study is that E2 failed to increase eNOS and ERα expression after 9 months of estrogen deprivation. Consequently, E2 failed improving endothelium-dependent dilation. In all groups, endothelium-dependent dilation was totally suppressed by N(omega)-nitro-L-arginine methyl ester showing that it was fully dependent on NO synthesis. Our observation is in agreement with a previous study showing that prolonged ovariectomy (18 months) in the rat shows that prolonged ovariectomy (18 months) in the rat induces a reduction in NO-dependent relaxation in the tail artery. Furthermore, the present study provides a possible explanation for the observations made in estrogen treatment in monkeys started shortly after the establishment of estrogen deficiency prevents atherosclerosis whereas this protective effect is lost when the treatment is delayed.

Because the loss of function in rats submitted to a 9-month estrogen deprivation was associated with a 50% reduction in ERα expression level, we submitted ERα−/− mice to a chronic increase in blood flow in vivo and found that this was associated with a 50% reduction in remodeling. This result shows that FMR is sensitive to ERα gene dosage.

Finally, in rats submitted to prolonged ovariectomy, treated or not with E2, endothelium-independent relaxation induced by the NO donor sodium nitroprusside was higher than in intact rat, suggesting an improved sensitivity to NO of the vascular smooth muscle cells, most probably to compensate for the reduced production of NO by the endothelium as evidenced by a diminished acetylcholine-dependent relaxation. This in agreement with previous studies showing that the chronic treatment with the NO synthesis blocker N(omega)-nitro-L-arginine methyl ester was associated with increased sensitivity of the uterine artery, in the aorta, and in the mesenteric resistance artery.

Pathophysiological Consideration

Our study brings new insights into the role of estrogens on long-term vascular homeostasis. Besides confirming the key role of E2 and ERα in FMR of resistance arteries, we demonstrated experimentally the correlation between E2 blood level and the amplitude of the remodeling in response to a chronic increase in blood flow. This remodeling has a key role in postischemic revascularization, and our finding could provide an explanation, at least in part, for the protective effect of estradiol in women aged >65 years. Indeed, older women with higher level of endogenous estradiol remain better protected against ischemic arterial disease than women with lower endogenous estradiol level.

Interestingly, we also found that a prolonged estrogen deprivation induced a loss of the ability of the arteries to adapt in response to a chronic increase in blood flow, thus bringing a new insight in our understanding of the timing effect. Indeed, the downregulation of ERα could be a crucial event of the timing effect that should be taken into account to better understand the lack of protection of hormonal substitutive treatment described in the HERS and in the WHI study.

Conclusion

Our study showed that aging in female rats was associated with a progressive reduction in both FMR and blood E2. Prolonged estrogen deprivation induced a loss of the ability of E2 to restore remodeling in association with reduced ERα and eNOS expression level, providing a possible explanation for the timing effect in the failure of delayed estrogens to confer vascular protection.

Acknowledgments

We thank Celine Beaujean for taking care of the animals. We thank Andrée Krust and Pierre Chambon for kindly providing ERα−/− mice.

Sources of Funding

The work performed in Angers (BNMI) was supported in part by the Foundation for Medical Research (Paris, France), the Fondation de France, Angers-Loire Metropole, Département du Maine et Loire, and the Region Pays-de-la-Loire. The work performed in Toulouse (INSERM 1048) was supported by Fondation pour la Recherche Medicale, Fondation de France, and Région Midi-Pyrénées.

Disclosures

None.

References


31. Loufrani L and Henrion D. Reactive oxygen species are necessary for high shear stress-mediated remodeling and endothelial nitric oxide synthase expression. Thus, E2 deprivation, more than age, reduced flow-mediated remodeling. However, delayed replacement of 9 months impaired E2 action, underlining the importance of timing in E2 protective effect.

**Significance**

Flow (shear stress)-mediated outward remodeling of resistance arteries has a major role in collateral growth after arterial occlusion but declines rapidly with age in male rats. Because 17β-estradiol (E2) is essential in this process, we investigated the impact of age and timing for estrogen efficacy on remodeling in female rats 3 to 18 months old. The amplitude of remodeling declined slowly with age in parallel with E2 blood level. E2 substitution failed restoring remodeling in 18-month-old rats. Ovariectomy of 3- to 12-month-old rats abolished remodeling, which was restored by immediate E2 replacement. This effect of E2 was abrogated if E2 replacement was delayed by 9 months in association with reduced estrogen receptor α and endothelial nitric oxide synthase expression. Thus, E2 deprivation, more than age, reduced flow-mediated remodeling. However, delayed replacement of 9 months impaired E2 action, underlining the importance of timing in E2 protective effect.
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Arteriosclerosis, Thrombosis, and Vascular Biology. published online April 3, 2014;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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1. Animal Protocol

1.1. Female rats:

Three- to 18-month old female Wistar rats (Charles River, L’Arbresles, France) were ovariectomized (OVX) under isoflurane (2.5%) anesthesia, as previously described\(^1\). Uterus were collected at the end of each protocol and weighted in order to control the effectiveness of the ovariectomy. All rats were purchased at the age of 3 months and then housed in control conditions and received food and water ad libitum. Rats were allowed at least 2 weeks to adapt to the animal facility before inclusion in a protocol.

After 1 week, rats were submitted to surgery in order to increase blood flow in 1 mesenteric artery as previously described\(^2\). Three consecutive first-order arteries were used. Ligatures were applied to second-order branches (Figure below). The artery located between the two ligated arteries was designed as high flow (HF) artery. Arteries located at distance of the ligated arteries were used as control (normal flow, NF). Location of the ligations is shown below:

![Diagram of surgery applied to rat mesenteric resistance arteries](image)

Schematic representation of the surgery applied to rat mesenteric resistance arteries in order to increase locally arterial blood flow in one vessel. Second order mesenteric arteries where ligated (L = ligature) on 2 branches of the mesenteric circulation. The artery located between the ligated branches was thus submitted to a chronic increase in flow (high flow: HF). Similar arteries located at distance in the mesenteric vascular bed were used as control (NF, normal flow) arteries.

The following groups were used (12 rats were used per group):

1. Control (NF, normal flow)
2. High flow (HF)
3. First order (1\(^st\) order) arteries with ligations
4. Second order branches (2\(^nd\) order) without ligations
a- Three- to 18-month old intact female Wistar rats were submitted to surgery in order to change blood flow in mesenteric arteries. Vessels collected after 2 weeks for diameter measurement of HF and NF arteries (n=12 rats per group).

b- Eighteen-month old female rats treated or not with 17β-estradiol (E2, 20µg/kg per day, osmotic minipump, 3 weeks). Mesenteric arteries were ligated after one week (n=12 rats per group). NF and HF arteries were then collected after 2 weeks for diameter measurement.

c- Three-month old female rats were divided into 3 groups: control, OVX, OVX treated with 17β-estradiol (E2, 20µg/kg per day, osmotic minipump, 3 weeks). After one week, rats were submitted to surgery (mesenteric arteries ligation). NF and HF arteries were collected after 2 weeks for analysis.

d- Four other groups of 3-month-old female rats (n=12 rats per group) were ovariectomized. After 3 or 9 months they were treated with E2 (20µg/kg per day, osmotic minipump, 3 weeks) or not and one week later mesenteric arteries were ligated. Arteries were collected 2 weeks latter for analysis.

e- The 4 last groups of 3-month-old female rats (n=12 rats per group) were left 3 or 9 months before being ovariectomized and treated or not with 17β-estradiol (E2, 20µg/kg per day, osmotic minipump, 3 weeks). One week later mesenteric arteries were ligated. NF and HF arteries were collected after 2 other weeks.

Schematic representation of the experimental protocol applied to female rats aged 3 to 12 months. Rats were ovariectomized (OVX) or not one week before ligating mesenteric arteries. Arteries were collected 2 weeks after ligation of analysis. This protocol was performed in 3, 6 and 12-month old rats. In some groups ovariectomy was performed 3 or 9 months before ligating arteries.

In each protocol, rats were anesthetized with isoflurane (2.5%). They were treated with buprenorphine (Temgesic®, 0.1 mg/kg, s.c.) before and after surgery.
Before harvesting the mesenteric arteries, arterial blood pressure was measured as previously described. Blood was then collected for E2 level measurement using a commercially available kit (Estradiol EIA Kit#58225 Cayman Chemical).

Rats were then sacrificed in a CO₂ chamber. The mesentery was quickly removed and placed in an ice-cold physiological salt solution (PSS) of the following composition (in mmol/L): 135.0, NaCl, 15.0, NaHCO₃, 4.6 KCl, 1.5, CaCl₂, 1.2, MgSO₄, 11.0, glucose, 10.0, N-2-hydroxyethylpiperazine-N-2-ethyisulfonic acid. The PSS was maintained at pH 7.4, PO₂ 160 mmHg, PCO₂ 37 mmHg. Mesenteric arteries (HF and NF) were gently dissected and divided into two segments, proximal for the functional study and distal for histological and biochemical studies.

1.2. Protocol applied to mice:

Three-month-old female mice were submitted to surgery on mesenteric arteries in order to increase chronically blood flow in HF arteries (protocol as described above). Mice on a C57BL/6J background were homozygous (-/-) or heterozygous (+/-) animal lacking the gene encoding for ERα. They were compared with their littermate wild-type (WT or +/-). Mice were anesthetized with isoflurane (2.5%) and treated with buprenorphine (Temgesic®; 0.1 mg/kg, s.c.) before and after surgery. Mice were then sacrificed in a CO₂ chamber before harvesting the mesentery. Height mice were used per group.

1.3. Ethical consideration:

The investigation conforms to the European Community standards on the care and use of laboratory animals (authorization nb 00577) and to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The protocol was approved by the ethical committee (Protocol CEEA PdL #2008.10).

2. Pressure-diameter relationship in mesenteric arteries in vitro

Arterial segments were cannulated at both ends and mounted in a video monitored perfusion system (Living System, LSI, Burlington, VT) as previously described. Arterial segments were bathed in a 5 ml organ bath containing a Ca²⁺-free PSS containing ethylene-bis-(oxyethylenenitrolo) tetra-acetic acid (EGTA, 2 mmol/L) and sodium nitroprusside (SNP, 10 µmol/L). Pressure steps (10 to 150 mmHg) were then performed in order to determine passive arterial diameter. Pressure and diameter measurements were collected using a Biopac data acquisition system (Biopac MP100 and Acqknowledge® software; La Jolla, CA, USA)².

3. Endothelium-dependent relaxation

Segments of NF and HF arteries (2-mm long each) were dissected and mounted in a wire myography (DMT)⁸. Cumulative concentration-response curves to acetylcholine (0.01 to 10 µmol/L) were performed before and after incubation (20 minutes) with the NO-synthase inhibitor L-NAME (10µmol/L). Acetylcholine-dependent relaxation was performed after precontracted of the arteries with phenylephrine (0.1 µmol/L) and serotonin (0.1 µmol/L) to
approximately 70% of their maximal contractile response.

4. Western-blot analysis in HF and NF arteries:

As previously described, segments of HF and NF arteries were quickly frozen and then pulverized in liquid nitrogen. The sample powders obtained were resuspended in lysis buffers. Vessel extracts were incubated on ice for 30 minutes and then centrifuged (14,000 rpm, 20 minutes at 4°C). Proteins (30 mg total protein from each sample) were separated by 10% SDS-PAGE and transferred to nitrocellulose. These membranes were subsequently incubated overnight with the primary antibody before being incubated with the appropriate peroxidase-labeled secondary antibody for 1 h. The reaction was visualized by ECL detection according to the manufacturer's instructions (Amersham Biosciences, GE, CT). Membranes were stripped in 0.1 M glycine, pH 2.2, for 1 h before reblotting. The anti-ERalpha polyclonal antibody (MC-20, catalog # sc-542) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-eNOS antibody (catalog # 610297), anti-ERK antibody (catalog # 610031), anti-gp91 phox (catalog # 611415) and anti-p67 phox (catalog # 610913) antibodies were from BD Biosciences (BD, NJ). The mAb-specific for Beta actin (catalog # A5316) was bought from Sigma-aldrich (Sigma, MO). Antibody rose against phospho-ERK (catalog # 9101) and anti-AKT (catalog # 9272) were bought from Cell Signaling (MA). The anti-EC-SOD (catalog # SOD-106) and Mn-SOD (catalog # SOD-110) were purchased from Enzo Life Sciences (NY). Goat anti-mouse and anti-rabbit peroxidase-labeled immunoglobulins were from Thermoscientific (Thermo Fisher Scientific, MA).

5. Histomorphometric analysis

Arterial segments pressurized at 75 mmHg and fixed in a 4% buffered formaldehyde solution were cut using a cryostat. Transverse sections (7 μm thick) were stained with orcein solution. The external diameter, lumen diameter and media thickness were determined after image acquisition (Olympus T100 microscope Olympus France, Rungis, France, Sony camera, Sony, Rungis, France) and analyzed using the Histolab software (Microvision, Paris, France) for calculations of cross-sectional area.

6. Statistical Analysis

Results were expressed as means±SEM. Significance of the differences between groups was determined by analysis of variance (ANOVA for consecutive measurements for pressure-diameter curves) or 1-way ANOVA followed by Bonferroni. Probability values less than 0.05 were considered significant.

7. References


Supplement figure I: Arterial diameter measured in response to stepwise increases in pressure in mesenteric arteries submitted chronically to high flow (HF) or to normal flow (NF). Arteries were isolated from 3-months old intact rats (control, A), ovariectomized rats (B) and ovariectomized rats treated with 17-b-estradiol (E2) (C). Mean ± sem is represented (n=12 rats per group).

*P<0.05, HF versus NF arteries.
Supplemental figure II:
Arterial diameter measured in response to stepwise increases in pressure in mesenteric arteries submitted chronically to high flow (HF) or to normal flow (NF). Arteries were isolated from 6-months-old female rats. Rats were intact rats (A), rats ovariectomized (OVX) at the age of 6 months (B) and treated with E2, rats ovariectomized at the age of 3 months (C) and rats ovariectomized at the age of 3 months and treated with 17β-estradiol (E2) at the age of 6 months. Mean ± sem is represented (n=12 rats per group).

*P<0.05, HF versus NF arteries.
Supplement figure III:
Ratio of the protein expression level of phospho-eNOS to eNOS in mesenteric arteries isolated from 12-month-old rats ovariectomized at the age of 12 months (OVX) and treated or not with E2 at the age of 12 months. Rats were also 12-month-old rats ovariectomized at the age of 3 months (9 months OVX) treated or not with E2 at the age of 12 months.
Mean ± sem is represented (n=12 rats per group).
Supplement figure IV:
Protein expression level of ERK1/2 and Akt determined in mesenteric arteries isolated from 12-month-old rats ovariectomized at the age of 12 months (OVX) and treated or not with E2 at the age of 12 months. Rats were also 12-month-old rats ovariectomized at the age of 3 months (9 months OVX) treated or not with E2 at the age of 12 months. The ratio of phospho-ERK to ERK1/2 is shown in panel B. The ratio of protein expression in HF arteries to that measured in NF vessels is shown in panel C. Mean ± sem is represented (n=12 rats per group).

*P<0.05.
Supplement figure V:
Protein expression level of Akt (A) and phospho-Akt (B) determined in mesenteric arteries isolated from 12-month-old rats ovariectomized at the age of 12 months (OVX) and treated or not with E2 at the age of 12 months. Rats were also 12-month-old rats ovariectomized at the age of 3 months (9 months OVX) treated or not with E2 at the age of 12 months. The ratio of protein expression in HF arteries to that measured in NF vessels is shown in panel C.
Mean ± sem is represented (n=12 rats per group).
*P<0.05.
Supplement figure VI: Protein expression levels of EcSOD and MnSOD determined in mesenteric isolated isolated from 12-months old rats ovariectomized at the age of 12 months (OVX) and treated or not with E2 at the age of 12 months. Rats were also 12-month-old rats ovariectomized at the age of 3 months (9 months OVX) treated or not with E2 at the age of 12 months. The ratio of protein expression in HF arteries to that measured in NF vessels is shown in panel B. Mean ± sem is represented (n=12 rats per group).

*P<0.05, versus 12-month old rats (blue)
Supplement figure VII: Protein expression level of p67 phox determined in mesenteric isolated from 12-months old rats ovariectomized at the age of 12 months (OVX) and treated or not with E2 at the age of 12 months. Rats were also 12-months old rats ovariectomized at the age of 3 months (9 months OVX) treated or not with E2 at the age of 12 months. The ratio of protein expression in HF arteries to that measured in NF vessels is shown in panel B. Mean ± sem is represented (n=12 rats per group).

*P<0.05, versus 12-month old rats (blue)
Supplement figure VIII: Protein expression level of gp91 phox (A) and p22 phox (B) determined in mesenteric isolated from 12-months old rats ovariectomized at the age of 12 months (OVX) and treated or not with E2 at the age of 12 months. Rats were also 12-months old rats ovariectomized at the age of 3 months (9 months OVX) treated or not with E2 at the age of 12 months.

The ratio of protein expression in HF arteries to that measured in NF vessels is shown in lower panels. Mean ± sem is represented (n=12 rats per group).

*P<0.05, versus 12-month old rats (blue)
Supplement figure IX: Protein expression level of TNFα (A), COX-2 (B) and HO-1 (C) determined in mesenteric isolated from 12-months old rats ovariectomized at the age of 12 months (OVX) and treated or not with E2 at the age of 12 months Rats were also 12-months old rats ovariectomized at the age of 3 months (9 months OVX) treated or not with E2 at the age of 12 months. The ratio of protein expression in HF arteries to that measured in NF vessels is shown in the right panels. Mean ± sem is represented (n=12 rats per group).