Estrogen and Ischemic Brain Injury

Activation of Estrogen Receptor-α and of Angiotensin-Converting Enzyme 2 Suppresses Ischemic Brain Damage in Oophorectomized Rats

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See Editorial Commentary, pp 1056–1057

Abstract—Like the angiotensin II type 1 receptor blocker, endogenous estrogen (17β-estradiol) is neuroprotective against cerebral ischemia; its effects are thought to be mediated by estrogen receptors (ERs). To verify the role of ERs and the brain renin-angiotensin system in estrogen-deficient rats with ischemia induced by middle cerebral artery occlusion, we compared rats subjected to oophorectomy (OVX+) with sham-oophorectomized rats (OVX−) and OVX+ rats treated with 0.3 or 3.0 mg/kg of olmesartan for 2 weeks before middle cerebral artery occlusion. Independent of the blood pressure, the cortical infarct volume was larger in OVX+ than in OVX− rats. It was smaller in olmesartan-pretreated OVX+ rats. The expression of ERα in the peri-infarct region was correlated with the reduction of cortical infarct but not that of ERβ or G protein–coupled estrogen receptor. Olmesartan prevented ERα downregulation in the cortical peri-infarct area, without affecting ERβ or G protein–coupled estrogen receptor. Olmesartan also increased mRNA expression of angiotensin-converting enzyme 2, Bcl-2, and Bcl-xL and reduced angiotensin II and cleaved caspase 3. These effects were augmented by olmesartan and abolished by the ER inhibitor. In OVX+ rats treated with the ERα agonist alone, the infarct size was decreased, and the neuroprotective genes were upregulated. These findings suggest that the transactivation of neuroprotective genes and the reduction in brain angiotensin II are ERα dependent and that this may augment neuroprotection together with an angiotensin II type 1 receptor blockade by olmesartan. We present the new insight that the activation of ERα independent of estrogen contributes at least partly to limiting cerebral ischemic damage.  

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Key Words: cerebral ischemia ■ estrogen receptor-α, estrogen receptor-β, G protein–coupled estrogen receptor ■ brain renin-angiotensin system ■ olmesartan ■ oophorectomy

The incidence of stroke is typically higher in middle-aged men than in women. Its increase in postmenopausal women is thought to be attributable to hypoestrogenicity.1–3 The binding of estrogen to nuclear receptors, estrogen receptor (ER)-α and ERβ, can alter the protein expression involved in neuroprotection; both ER subtypes contribute to vasoprotection in a cell type–specific manner.2–10 In addition to these “classic” ERs, the G protein–coupled ER (GPER) has been identified as a “nonclassic” receptor and shown to contribute to the rapid effects of estrogen.10,11 Although several studies examined the protective role of ERs against ischemic brain damage,4,5,11,12 the precise role of each ER remains obscure, especially under conditions of estrogen deficiency.

Activation of the renin-angiotensin system (RAS) predisposes to atherosclerosis and thromboembolic events and is involved in ischemic brain damage.13 Angiotensin (Ang) II exerts blood vessel damaging actions; they are mediated mainly by the Ang II type 1 receptor (AT1R). The Ang II type 2 receptor (AT2R) is thought to counteract AT1R-mediated effects and to exert protective effects against ischemia.14 Elsewhere we reported that, in male rats, the Ang II type 1 receptor blocker (ARB) exerts neuroprotective effects against cerebral ischemia; it upregulated endothelial NO synthase in an AT,R-dependent- and blood pressure–independent manner.15 We also showed that the endothelial expression of AT1R and AT2R was modulated by estrogen and that the vasoprotective effects of ARB were augmented in the presence of estrogen.16 However, there are few studies on the efficacy of ARB against cerebral ischemic damage and its mechanisms under estrogen-deficient conditions.

Renin converts angiotensinogen into Ang I, and Ang-converting enzyme (ACE) converts Ang I into Ang II. ACE2, a homologue of ACE, converts mainly Ang II into the Ang-(1-7) peptide that binds to the Mas receptor to stimulate vasodilation and potentiate bradykinin; it thereby exerts cardioprotective effects.17 Although the presence of ACE2 protein and mRNA in
Before MCAO, the blood pressure in OVX+ rats treated with 0.3 but not with 3.0 mg/kg of olmesartan was similar to the blood pressure in OVX+/VC rats. During and after MCAO it was lower in olmesartan-treated than OVX+/VC rats (Table S1). The infarct volume in the cortex but not the basal ganglia was reduced in rats treated with 0.3 mg/kg (P<0.05) and 3.0 mg/kg (P<0.01; Figure 1). The reduction in the infarct volume in rats treated with 0.3 mg/kg of olmesartan appeared to be independent of their preischemic blood pressure. Because the cerebral blood flow was not different in any rats of all of the groups (data not shown), the suppression of a blood pressure increase during and after MCAO suggests that olmesartan improved cerebral blood flow autoregulation in OVX+ rats as it did in male rats.15

**ERα Downregulation and RAS Activation After Brain Ischemia in OVX+ Rats Were Suppressed by Olmesartan**

Based on the suggestion20 that the presence of a penumbra may render therapeutic salvage possible, we focused on the role of ER and the brain RAS in the cortical peri-infarct area. Immunohistochemically, ERα-positive neurons and endothelia (Figure S1A) were increased in the ischemic penumbra of OVX+/VC rats (Figure 2A) compared with the nonischemic contralateral side (Figure S1B); this increase was lower in OVX+/VC rats and enhanced by olmesartan treatment (Figure 2A and 2B). The mRNA level of ERα was correlated with protein expression (Figure 3A). On the other hand, the constitutive gene and protein expressions of ERβ and GPER were detected on both sides of the brain and not affected by OVX and olmesartan (Figure S1C and S1D). Correlated with the reduced ERα expression, in OVX+/VC rats the transcriptional activation of microtubule-associated protein (MAP) 2, Bcl-2, and Bcl-xL as survival- and antiapoptosis-related genes was lower than in OVX+/VC rats (Figure 3B through 3D). The expression of cleaved caspase 3 was increased in OVX+/VC rats (Figure 2A and 2C). These molecular changes were reversed by olmesartan (Figures 2A, 2C, and 3B through 3D).

The other hand, the protein expression of Ang II and AT1-R was higher in OVX+/VC than OVX+/VC rats (Figure 2A, 2D, and 2E); the expression of AT2-R was lower in OVX+/VC rats (Figure 2A and 2F). Their expression was mainly observed in neurons and endothelial cells of the penumbra after MCAO (Figure S2A through S2C). The mRNA level of AT1-R and AT2-R was correlated with their protein expression (Figure 3E and 3F). In OVX+/VC rats, ACE2 mRNA but not ACE mRNA was decreased (Figure 3G and 3H). Olmesartan also restored these phenomena in OVX+/VC rats (Figures 2A and 2D through 2F and 3E, 3F, and 3H). These results suggest that estrogen deficiency induced by OVX downregulated ERα and promoted the increase in Ang II and AT1-R expression. Olmesartan may limit the infarct size through the upregulation of ERα and of the neuroprotective molecules and by inhibiting brain RAS activation.

**Neuroprotection by Olmesartan Was Partly Dependent on ERα**

To further confirm that the activation of ERα in the estrogen-deficient state is associated with the induction of neuropro-

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**Materials and Methods**

For a detailed description of Materials and Methods, see the online Data Supplement at http://hyper.ahajournals.org.

**Results**

**The Cortical Infarct Volume, Increased in Estrogen-Deficient Rats Without Affecting the Blood Pressure, Was Reduced by Olmesartan**

Before, during, and after middle cerebral artery occlusion (MCAO), the systolic, mean, and diastolic blood pressures were similar in oophorectomized (OVX+/VC) and sham-oophorectomized (OVX+/VC) rats serving as the vehicle controls (Table S1, available in the online Data Supplement at http://hyper.ahajournals.org). In OVX+/VC rats, the cortical but not the basal ganglia infarct volume was significantly larger than in OVX+/VC rats (Figure 1), suggesting that estrogen deficiency by OVX blood pressure independently exacerbates the ischemic insult.
tective molecules, we administered ER inhibitor (ERI) ICI 182 780 to rats treated with 3.0 mg/kg of olmesartan. ERI partially abolished the olmesartan-induced reduction in the infarct volume (Figure 4A). The olmesartan-induced increase in ACE2 mRNA was reversed by ERI (Figure 4E). However, neither the olmesartan-induced downregulation of AT1R nor the upregulation of AT2R was affected by ERI (Figure S3A and S3B). ERI treatment increased the expression of Ang II (Figure 4B), indicating that the upregulation of ACE2 by olmesartan is mediated by ERα, resulting in the reduction of brain Ang II expression. The expression of cleaved caspase 3 was increased by ERI (Figure 4C); the olmesartan-induced elevation in the mRNA level of Bcl-2 and Bcl-xL but not of MAP2 was abrogated by ERI (Figures 4F, 4G, and S3C).

The serine 118 residue (Ser-118) in ERα is a major site of phosphorylation in response to its ligand. Phosphorylated ERα influences the recruitment of coactivators of transcription. The expression of phosphorylated and total ERα was increased by olmesartan and decreased by ERI (Figure 4D), suggesting that the transcriptional activation of antiapoptotic genes is associated with the olmesartan-induced upregulation of phosphorylated ERα.

To further confirm the functional role of ERα on neuroprotection after ischemia, we treated OVX+VC rats with the ERα agonist propyl pyrazole triol. The infarct volume was reduced, and the mRNA level of ACE2, Bcl-2, and Bcl-xL was upregulated in the peri-infarct area of propyl pyrazole triol–treated compared with nontreated rats (Figure 5A through 5D), suggesting an essential role of ERα activation after ischemia.

Discussion

We present new insights into neuroprotection after cerebral ischemia in OVX+ rats. First, we demonstrated that estrogen deficiency exaggerated ischemic brain damage and that ERα activation was associated with neuroprotection against ischemic damage independent of estrogen. Olmesartan augmented the activation of ERα and suppressed brain damage in OVX+ rats independent of the preischemic blood pressure. Second, we found that the activation of ERα induced the transcriptional activation of antiapoptotic Bcl-2 and Bcl-xL, resulting in the reduction of cleaved caspase 3. More importantly, it upregulated ACE2 and reduced Ang II; the upregulation of ACE2 and antiapoptotic molecules was ERα dependent, whereas the regulation of AT1R, AT2R, and MAP2 was not. These findings suggest that ERα activation is essential for and contributes at least partly to neuroprotection after ischemia under estrogen-deficient conditions and that olmesartan has ERα-dependent and -independent effects in addition to the blockade of AT1R.

Figure 2. Representative immunohistochemical staining (A) for ERα, Casp-3, Ang II, AT1R, and AT2R. Western blot analysis for the expression of ERα (B), Casp-3 (C), Ang II (D), AT1R (E), and AT2R (F). All of the values were normalized by setting the densitometry of the control sample to 1.0 (each group n=6). Each bar represents the mean±SD; **P<0.01 vs OVX+VC by ANOVA followed by Scheffe test. Ang II indicates angiotensin II; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; Casp-3, cleaved caspase-3; ER, estrogen receptor; olm, olmesartan; OVX, oophorectomy; VC, vehicle control.
ERα is essential in estradiol-mediated protection against cell death in the brain.\(^4^,5\) We showed that, under estrogen-deficient conditions, the olmesartan-induced upregulation of ERα was associated with the upregulation of antiapoptotic genes, resulting in a reduction in the cortical infarct size. Previous reports demonstrated ERβ-dependent neuroprotection after ischemia\(^12\) and that GPER activation was associated with acute vasoprotective and neuroprotective effects.\(^10\) Other studies showed that ERβ was irrelevant to neuroprotection and that GPER led to enhanced apoptosis via extracellular signal-regulated kinase activation.\(^5,22\) Because we found that neither estrogen nor olmesartan had an effect on the protein and mRNA expression of ERβ and GPER, we cannot state that ERβ and GPER protect against ischemic damage.

Several specific mechanisms are involved in the modulation of ERα expression.\(^5,23,24\) In female rats, ischemia led to demethylation of the ERα promoter, resulting in an increase in the expression of ERα.\(^4\) The expression of ERα on ischemia was enhanced by olmesartan even in OVX\(^+\) rats. The stabilization of ERα or the promotion of its demethylation by olmesartan may be related to the increase in ERα under estrogen-deficient and ischemic conditions. Additional studies are necessary to elucidate more details on the regulation of ERα expression.

ERα contains the N-terminal ligand-independent activation function (AF1)-, the DNA binding-, the hinge-, and the C-terminal ligand binding domain that contain a ligand-regulated activation function (AF2).\(^21,25\) In response to estrogen, AF1 synergizes with AF2. Thus, AF1 plays a role in both ligand-dependent and -independent transcription. In addition, Ser-118 in ERα is located within the AF1 region and is phosphorylated in response to the activation of cellular kinases by extracellular signals.\(^21\) The olmesartan-induced elevation of phosphorylated ERα would be involved in the transcriptional activation of neuroprotective genes via a ligand-independent pathway associated with AF1.

In animals treated with ARB, ACE2 expression was increased in the heart and kidney.\(^18\) We first observed that, in OVX\(^+\) rats treated with olmesartan, the transcriptional activity of ACE2 was increased through the activation of ERα in the cerebral ischemic penumbra. The upregulation of ACE2 via ERα activation by olmesartan may reduce Ang II expression and thereby contribute at least partly to limiting brain damage after ischemia.

Compared with OVX\(^−\) rats, the expression of AT\(_1\)R was decreased and the expression of AT\(_2\)R was increased in OVX\(^+\) rats; these were similar to the effects elicited by olmesartan. These data suggest that neuroprotection by not only olmesartan but also by endogenous estrogen may be associated with both AT\(_1\)R and AT\(_2\)R expression. This result is partially consistent with an earlier report.\(^16,26\) However,
ERα activation by olmesartan did not affect the transcriptional regulation of AT1R, AT2R, and MAP2, suggesting that the ligand-dependent pathway may be different from the ligand-independent pathway of transcription. Li et al. demonstrated AT2R-dependent neuronal survival with the increase in MAP2 by ARB after ischemia. Not only AT1R blockade but also the reduction of Ang II via increased ACE2 could lead to AT1R downregulation and to the upregulation of AT2R linked to MAP2. Thus, AT2R stimulation would also partly contribute to the observed neuroprotection.

Figure 4. Effects of the estrogen receptor inhibitor (ERI) on neuroprotection induced by olmesartan. ERI (3.0 mg/kg) was administered to rats treated with 3.0 mg/kg of olmesartan 30 minutes before middle cerebral artery occlusion (MCAO). The olmesartan-induced reduction in the infarct volume (A, each group n=8) and the protein expression of Ang II (B) and Casp-3 (C) were abolished by ERI (each group n=6). The expression of phospho- and total ERα was increased by olmesartan and decreased by ERI (D, each group n=6). The olmesartan-induced elevation in the mRNA level of ACE2 (E), Bcl-2 (F), and Bcl-xL (G) was abrogated by ERI (each group n=8). Each bar represents the mean±SD; *P<0.05, **P<0.01 vs OVX/VC, #P<0.05, ##P<0.01 vs OVX/olm 3.0 mg/kg by ANOVA followed by Scheffe test. ACE2 indicates angiotensin-converting enzyme 2; Ang II, angiotensin II; Casp-3, cleaved caspase-3; olm, olmesartan; OVX, oophorectomy; pho-ERα, phosphorylated Ser-118 estrogen receptor α; VC, vehicle control.

ERα activation by olmesartan did not affect the transcriptional regulation of AT1R, AT2R, and MAP2, suggesting that the ligand-dependent pathway may be different from the ligand-independent pathway of transcription. Li et al. demonstrated AT2R-dependent neuronal survival with the increase in MAP2 by ARB after ischemia. Not only AT1R blockade but also the reduction of Ang II via increased ACE2 could lead to AT1R downregulation and to the upregulation of AT2R linked to MAP2. Thus, AT2R stimulation would also partly contribute to the observed neuroprotection.

Figure 5. Effects of the estrogen receptor (ERα) agonist propyl pyrazole triol (PPT) on cerebral ischemia in OVX rats. We treated OVX/VC rats with the ERα agonist PPT (2.0 mg/kg). In rats treated with PPT, the infarct volume was reduced (A), and the mRNA level of ACE2 (B), Bcl-2 (C), and Bcl-xL (D) was upregulated in the peri-infarct area compared with nontreated rats (each group n=8). Each bar represents the mean±SD. *P<0.05 vs OVX/VC by ANOVA followed by the Mann-Whitney U test. ACE2 indicates angiotensin-converting enzyme 2; olm, olmesartan; MCAO, middle cerebral artery occlusion; OVX, oophorectomy; VC, vehicle control.
Perspectives
We first demonstrate the regulation of ERα by ARB in vivo. Additional studies are underway to clarify whether ERα contributes to neuroprotection in male rats. Ovariectomized animals mimicking the status of postmenopausal women may serve as a model of "surgical menopause," a condition associated with the development of accelerated atherosclerosis and hypertension in humans. Because we used 13-week-old normotensive rats manifesting hypoestrogenicity for 4 weeks, our results may not reflect the possible effects of aging, hypertension, and prolonged ovarian hormone deprivation.

Although we cannot rule out other mechanisms underlying the neuroprotection by olmesartan, it may represent a therapeutic means for treating cerebrovascular damage in menopausal and cycling women. In clinical studies, important questions have been raised regarding the effects of estrogen on vascular inflammation. To demonstrate the benefits of olmesartan as an alternative to hormone replacement therapy and to elucidate its neuroprotective role in postmenopausal women, prudent clinical studies are needed.

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Disclosures
None.

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