

# Hyper-reactivity of cerebral arteries from ovariectomized rats: therapeutic benefit of tamoxifen

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**1** An increased incidence of systemic hypertension has been documented in postmenopausal women and identified as an independent risk factor in the development of cerebrovascular stroke. The present study examined whether cerebrovascular reactivity was increased in the hypertensive ovariectomized rat, and explored the potential therapeutic benefit of the partial estrogen receptor agonist tamoxifen.

**2** Female Sprague–Dawley rats were subjected to bilateral ovariectomy (OVX,  $n = 16$ ) or a sham operation ( $n = 8$ ). At 6-week postsurgery, rats were anesthetized to assess ventricular contractility and blood pressure. In a second series of experiments, OVX rats ( $n = 8$ ) were given tamoxifen starting 3 weeks postsurgery, and continued for 3 weeks. At the end of each protocol, the middle cerebral artery was harvested and rings were mounted in wire-myographs to measure isometric tension.

**3** Systolic arterial pressure (SAP) was significantly increased ( $P < 0.05$ ) in the OVX rat ( $174 \pm 8$  mmHg), as compared to sham ( $135 \pm 6$  mmHg). The resting tension of isolated cerebral arteries from OVX rats ( $186 \pm 15$  mg) was significantly elevated ( $P < 0.05$ ), as compared to sham ( $129 \pm 9$  mg). Phenylephrine treatment did not elicit a constriction of cerebral arteries isolated from sham rats, whereas a potent response ( $P < 0.05$ ) was observed in OVX rats. Nitric oxide (NO) synthase inhibition with L-NNA led to a limited contraction in sham rats ( $8 \pm 3\%$  of  $E_{\max}$ ), whereas a significant ( $P < 0.05$ ) increase was observed in OVX rats ( $34 \pm 12\%$  of  $E_{\max}$ ). Lastly, vascular sensitivity ( $pD_2$ ) to sodium nitroprusside was significantly increased ( $P < 0.05$ ) in OVX rats, as compared to sham.

**4** Tamoxifen therapy normalized the resting tension of isolated cerebral arteries from OVX rats, abrogated phenylephrine-mediated contraction, and modestly reduced SAP. By contrast, tamoxifen treatment of OVX rats did not attenuate L-NNA-mediated contractile response of cerebral arteries.

**5** These data demonstrate that the cerebral artery isolated from the OVX rat was associated with an exaggerated vasoconstrictor response to phenylephrine, and altered NO-dependent vascular reactivity. The administration of tamoxifen to OVX rats normalized cerebral artery reactivity to phenylephrine. These findings provide the impetus to examine the potential therapeutic benefit of the partial estrogen receptor agonist tamoxifen to reduce the incidence of cerebrovascular stroke in postmenopausal women.

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**Keywords:** Cerebral arteries; endothelin-1;  $\alpha_1$ -adrenergic receptors; nitric oxide; tamoxifen; ovariectomy

**Abbreviations:** DAP, diastolic arterial pressure; HRT, hormonal replacement therapy; L-NNA, *N*-nitro-L-arginine; LVEDP, left ventricular end diastolic pressure; LVSP, left ventricular systolic pressure; MCA, middle cerebral arteries; NO, nitric oxide; OVX, ovariectomized; SAP, systolic arterial pressure; SNP, sodium nitroprusside

## Introduction

Cerebrovascular stroke and coronary artery disease are two salient complications associated with menopause (Matthews *et al.*, 1989; Greendale *et al.*, 1999; Simon *et al.*, 2001). An increased incidence of systemic hypertension has been documented in postmenopausal women and identified as an independent risk factor in the development of cerebrovascular stroke and coronary artery disease (Kannel *et al.*, 1976; Stokes *et al.*, 1987; Laurent *et al.*, 2001; Reckelhoff, 2001). Based on these findings, hormonal replacement therapy (HRT; estrogen

and progesterone regiment) in postmenopausal women has received considerable interest. However, at least two independent clinical studies have shown that long-term HRT in either normal postmenopausal women or those with established coronary heart disease failed to demonstrate an overall cardioprotective action or reduce the incidence of stroke (Hulley *et al.*, 1998; Simon *et al.*, 2001; Rossouw *et al.*, 2002). Moreover, these latter clinical studies further highlighted that HRT was associated with the increased incidence of thromboembolism, coronary artery disease, as well as invasive breast cancer (Hulley *et al.*, 1998; Rossouw *et al.*, 2002).

Tamoxifen and raloxifene are representative of a family of molecules designated as selective estrogen receptor modulators (SERM). These molecules interact with the estrogen receptor,

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but are pharmacologically different from estrogen *via* its ability to act either as an agonist or antagonist depending on the target tissue and hormonal milieu. Tamoxifen is an effective antagonist in the treatment of estrogen-dependent breast cancer, whereas in bone, both tamoxifen and raloxifene mimic the beneficial action of estrogen (MacGregor & Jordan, 1998). Akin to estrogen, tamoxifen treatment promoted coronary artery relaxation, acutely and chronically (Jiang *et al.*, 1991; Mugge *et al.*, 1993; Chester *et al.*, 1995; Sudhir *et al.*, 1995). *In vivo* studies have demonstrated that SERM therapy reduced identifiable cardiovascular risk factors, including serum cholesterol, LDL, and fibrinogen B (Bjarnason *et al.*, 1997; MacGregor & Jordan, 1998; Walsh *et al.*, 1998; Clarke *et al.*, 2001). Surprisingly, and in contrast to HRT, the incidence of coronary artery disease and fatal MI in breast cancer patients receiving tamoxifen were reduced, as compared to placebo (McDonald & Stewart, 1991; McDonald *et al.*, 1995). In the Multiple Outcomes of Raloxifene Evaluation (MORE) study, raloxifene treatment significantly reduced the incidence of cardiovascular events in a subset of women characterized with increased cardiovascular risk (Barrett-Connor *et al.*, 2001). Thus, SERM therapy may represent a surrogate approach to HRT in postmenopausal women.

A review of 14 prospective, randomized, controlled trials demonstrated a 42% risk reduction of stroke when the diastolic arterial pressure (DAP) was reduced by 5–6 mmHg (Collins *et al.*, 1990). The Systolic Hypertension in the Elderly Program (SHEP) study found that treating isolated systolic hypertension in the elderly reduced stroke by 36% (SHEP Cooperative Research Group, 1991). These studies support a link between hypertension and stroke, albeit whether a similar relationship exists between hypertension-related menopause and cerebrovascular reactivity remains undefined. We have previously demonstrated that mean arterial pressure was significantly elevated in the ovariectomized (OVX) female rat, and normalized following the administration of an ET<sub>A</sub> receptor antagonist (Mercier *et al.*, 2002). In this regard, the present study tested the hypothesis that cerebrovascular reactivity was altered in the hypertensive OVX female rat. Second, the potential therapeutic benefit of the partial estrogen receptor agonist tamoxifen on cerebral artery reactivity in the OVX rat was examined.

## Methods

### *Ovariectomy model*

Female Sprague–Dawley rats (9–11 weeks; Charles River, St Constant, Quebec, Canada) were anesthetized with a ketamine (50 mg kg<sup>-1</sup>)/xylazine (10 mg kg<sup>-1</sup>) mixture and underwent either a sham surgery or bilateral ovariectomy. In a parallel study, OVX rats (*n* = 8) were given tamoxifen (10 mg kg<sup>-1</sup> day<sup>-1</sup>) in standard rat chow starting 3 weeks postsurgery, and continued for 3 weeks. Previous studies have demonstrated that mean arterial pressure was elevated and significant uterine atrophy was evident in 3 week OVX rats (Mercier *et al.*, 2002). At 6 weeks postsurgery, rats were anesthetized and hemodynamic parameters assessed, as previously described (Mercier *et al.*, 2002). Briefly, rats were anesthetized with a ketamine (50 mg kg<sup>-1</sup>)/xylazine (10 mg kg<sup>-1</sup>) mixture. A microtip pressure transducer catheter (model SPR-407, 2F; Millar Instru-

ment, Houston, TX, U.S.A.) was inserted in the carotid artery and advanced into the left ventricle. Systolic arterial pressure (SAP) and diastolic arterial pressure (DAP) were measured in the carotid artery prior to entry into the left ventricle. The pressures were recorded on a Gould 2600S recorder (Gould Instruments, Valley View, OH, U.S.A.). The uterus (uterine horn) was excised, immediately weighed, and rat middle cerebral arteries (MCA) were subsequently harvested from the brain. The use and care of laboratory animals was according to the Canadian Council for Animal Care and approved by the Animal Care Committee of the Montreal Heart Institute.

### *Isometric tension recording in isolated cerebral arteries*

Following hemodynamic measurements, rat MCA were harvested from the brain and placed in ice-cold physiological salt solution (PSS) containing indomethacin (10 µmol l<sup>-1</sup>, cyclooxygenase inhibitor) and the following composition (mmol l<sup>-1</sup>): NaCl 130, KCl 4.7, KH<sub>2</sub>PO<sub>4</sub> 1.18, MgSO<sub>4</sub> 1.17, NaHCO<sub>3</sub> 14.9, CaCl<sub>2</sub> 1.6, EDTA 0.026, glucose 10 and aerated with 12% O<sub>2</sub>/5% CO<sub>2</sub>/83% N<sub>2</sub> (pH 7.4). Segments 2 mm in length were mounted on 17 µm tungsten wires in microvessels myographs (IMF, University of Vermont, VT, U.S.A.) as previously described (Thorin *et al.*, 1998b). Arterial segments were stretched (300 mg) and permitted to reach their resting tension. This resting tension was optimal to induce a stable and reproducible contraction to 40 mmol l<sup>-1</sup> KCl-PSS. Resting tension was not affected by a Ca<sup>2+</sup>-free PSS containing 3 mmol l<sup>-1</sup> EGTA and 3 µmol l<sup>-1</sup> sodium nitroprusside (SNP) (data not shown). To prepare K<sup>+</sup>-rich solutions, equimolar amounts of NaCl were replaced with KCl.

### *Statistical analysis*

Results are expressed as mean ± s.e. In all experiments, *n* represents the number of rats. Contractions are expressed as a percentage of *E*<sub>max</sub> obtained in the presence of 127 mmol l<sup>-1</sup> KCl-PSS at the end of each experiment. Relaxations are expressed as a percentage of the initial precontraction. Half-maximum effective concentrations (EC<sub>50</sub>) of agonist-induced responses were measured from each individual dose–response curve using a logistic curve-fitting program (Allfit<sup>®</sup>, Dr Deléan, University of Montréal). The pD<sub>2</sub> value is the negative log of the EC<sub>50</sub>. Statistical differences between means were determined by analysis of variance followed by a Scheffé's *F* test or a Student's *t* test when only two groups were compared. A probability value of <0.05 was accepted as significant for differences between groups of data. Hemodynamic measurements were analyzed by a two-way ANOVA and a significant difference was determined by the Scheffé's *F* test.

### *Chemicals*

The following drugs were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.) indomethacin, *N*-nitro-L-arginine (L-NNA), phenylephrine, SNP, tamoxifen. Endothelin-1 was purchased from American Peptide Company (Sunnyvale, CA, U.S.A.). All drugs were dissolved in PSS except for indomethacin that was dissolved in ethanol; final concentration of ethanol in the bath was 0.1% (vol vol<sup>-1</sup>). Solutions were prepared freshly every day and kept on ice.

## Results

### Body weight, uterus atrophy, hemodynamic measurements, and the effect of tamoxifen

In 6-week OVX rats, an increase in body weight and uterus atrophy was evident, as compared to sham rats (Table 1). The administration of tamoxifen to OVX rats reversed the body weight gain, and partially attenuated uterus atrophy (Table 1). OVX significantly ( $P < 0.05$ ) increased SAP and DAP (Table 1). Left ventricular systolic pressure (LVSP),  $dp/dt$  indices, and left ventricular end diastolic pressure (LVEDP) were significantly increased in the OVX female rat, as compared to sham (Table 1). Tamoxifen treatment of OVX rats normalized LVEDP,  $-dp/dt$ , modestly reduced LVSP,  $+dp/dt$ , SAP, and had no effect on DAP (Table 1).

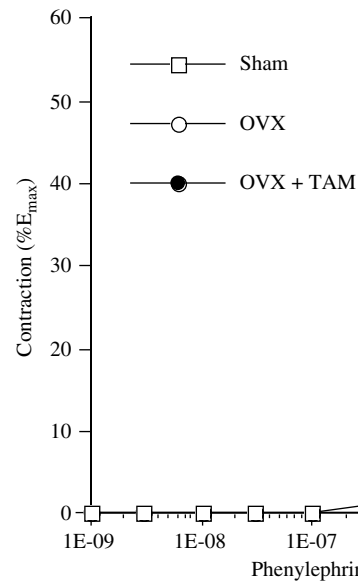
### Cerebral vascular reactivity and the effect of tamoxifen

At 6 weeks post ovariectomy, the resting tension and the  $E_{max}$  of isolated cerebral arteries were significantly ( $P < 0.05$ ) increased, and both parameters normalized with tamoxifen therapy (Table 2). The inhibition of nitric oxide (NO) synthase with L-NNA ( $100 \mu\text{mol l}^{-1}$ ) increased vascular tone in both groups, albeit the increase was quantitatively greater in the OVX rat, and was not reversed with tamoxifen treatment (Table 2). Lastly, a depolarizing PSS containing  $40 \text{ mmol l}^{-1}$  KCl induced a greater contraction of arteries isolated from OVX rats, as compared to sham rats, and tamoxifen therapy was without effect (Table 2).

Phenylephrine treatment did not elicit a contraction of the isolated rat cerebral artery in the sham rat with an intact endothelium (Figure 1). By contrast, the  $\alpha_1$ -adrenoceptor agonist induced a potent contraction ( $P < 0.05$ ) of the MCA in the OVX rat, and was prevented with tamoxifen therapy ( $P < 0.05$ ). Following the administration of L-NNA, phenylephrine induced a potent contraction in all groups with a maximal response representing  $57 \pm 6$ ,  $54 \pm 9$ , and  $68 \pm 8\%$  of  $E_{max}$  in sham-operated, OVX and OVX + tamoxifen rats,

respectively. The vascular sensitivity ( $pD_2$  values) to phenylephrine was increased in arteries isolated from OVX rats and not reduced by tamoxifen (Table 3).

Endothelin-1 induced a potent contraction of isolated cerebral arteries with an intact endothelium. The maximal contraction induced by the peptide was similar between sham-operated ( $105 \pm 5\%$  of  $E_{max}$ ), OVX ( $112 \pm 6\%$  of  $E_{max}$ ) and OVX + tamoxifen rats ( $102 \pm 3\%$  of  $E_{max}$ ). The sensitivity to endothelin-1 of arteries isolated from OVX rat was modestly increased and significant, as compared to arteries isolated from sham-operated rats (Table 3). This change in vascular sensitivity to endothelin-1 was reduced although not normalized by tamoxifen.



**Figure 1** Phenylephrine reactivity of cerebral arteries isolated from sham-operated (Sham;  $n = 8$ ), ovariectomized (OVX;  $n = 8$ ) rats, and tamoxifen-treated OVX (OVX + TAM;  $n = 8$ ) rats. Results are mean  $\pm$  s.e. \*:  $P < 0.05$  versus all other groups (ANOVA, Scheffé's  $F$  test).

**Table 1** Hemodynamic parameters, body and uterus weights of the sham, ovariectomized (OVX), and tamoxifen-treated OVX (OVX + TAM) rat

Groups	n	Body weight (g)	Uterus weight (g)	DAP (mmHg)	SAP (mmHg)	LVSP (mmHg)	$+dp/dt$ (mmHg s $^{-1}$ )	$-dp/dt$ (mmHg s $^{-1}$ )	LVEDP (mmHg)
Sham	8	280 $\pm$ 9	0.50 $\pm$ 0.02	96 $\pm$ 2	135 $\pm$ 6	116 $\pm$ 4	6327 $\pm$ 270	5331 $\pm$ 282	6.3 $\pm$ 0.8
OVX	8	362 $\pm$ 11*	0.11 $\pm$ 0.01*	114 $\pm$ 3*	174 $\pm$ 8*	150 $\pm$ 6*	7759 $\pm$ 360*	7032 $\pm$ 284*	10.1 $\pm$ 1.0*
OVX + TAM	8	278 $\pm$ 8†	0.22 $\pm$ 0.01*†	109 $\pm$ 3	150 $\pm$ 2	133 $\pm$ 14	6750 $\pm$ 267	5531 $\pm$ 300†	5.6 $\pm$ 0.6†

Results are expressed as mean  $\pm$  s.e. \*:  $P < 0.05$  versus Sham; †:  $P < 0.05$  versus OVX (ANOVA, Scheffé's  $F$  test).

**Table 2** The internal diameter (i.d.) and resting tension of isolated segments of MCA, and the contractile response to either  $127 \text{ mmol l}^{-1}$  KCl-PSS ( $E_{max}$ ),  $40 \text{ mmol l}^{-1}$  KCl-PSS ( $40 \text{ K}^+$ ), or  $N$ -nitro-L-arginine (L-NNA) of sham-operated (Sham), ovariectomized (OVX), and tamoxifen-treated OVX (OVX + TAM) rats

Groups	n	i.d. ( $\mu\text{m}$ )	Resting tension (mg)	$E_{max}$ (mg)	$40 \text{ K}^+$ (% $E_{max}$ )	L-NNA (% $E_{max}$ )
Sham	8	167 $\pm$ 5	129 $\pm$ 7	500 $\pm$ 35	53 $\pm$ 5	8 $\pm$ 3
OVX	8	178 $\pm$ 9	186 $\pm$ 15*	618 $\pm$ 51*	72 $\pm$ 4*	34 $\pm$ 12*
OVX + TAM	8	180 $\pm$ 9	142 $\pm$ 12†	525 $\pm$ 54†	76 $\pm$ 4*	23 $\pm$ 12

Results are expressed as mean  $\pm$  s.e. All PSS contained indomethacin ( $10 \mu\text{mol l}^{-1}$ ). \*:  $P < 0.05$  versus Sham; †:  $P < 0.05$  versus OVX (ANOVA, Scheffé's  $F$  test).

**Table 3** Sensitivity ( $pD_2$ ) of cerebral arteries isolated from sham-operated (Sham), ovariectomized (OVX), and tamoxifen-treated OVX (OVX+TAM) rats to phenylephrine (in the presence of L-NNA), endothelin-1, and SNP

Groups	Phenylephrine	Endothelin-1	SNP
Sham	6.00 ± 0.26	8.30 ± 0.09	6.23 ± 0.18
OVX	6.61 ± 0.15*	8.60 ± 0.08*	6.72 ± 0.16*
OVX + TAM	6.67 ± 0.16*	8.40 ± 0.06	6.66 ± 0.13*

Results are expressed as mean ± s.e. All PSS contained indomethacin ( $10 \mu\text{mol l}^{-1}$ ). \*:  $P < 0.05$  versus Sham (Student's *t* test).

SNP-induced maximal relaxation was similar between sham ( $79 \pm 6\%$ ), OVX ( $89 \pm 3\%$ ) and OVX + tamoxifen ( $90 \pm 4\%$ ) rats. However, the vascular sensitivity to SNP was increased in arteries isolated from OVX rats and not normalized with tamoxifen therapy (Table 3).

## Discussion

Clinical studies support the premise that postmenopausal women were associated with an increased incidence of stroke. Moreover, hypertension was prevalent in postmenopausal women, and represents an independent risk factor in the development of cerebrovascular stroke and coronary artery disease. Based on these observations, cerebrovascular reactivity may be altered during the progression of menopause, and thus may represent an underlying event contributing to the increased incidence of stroke. The present study has demonstrated that elevated blood pressure (SAP and DAP) in the OVX rat was associated with increased reactivity in response to contractile agents, phenylephrine ( $\alpha_1$ -AR agonist) and L-NNA (NO synthase blocker). Interestingly, whereas tamoxifen therapy abrogated phenylephrine-mediated vasoconstriction, and modestly reduced SAP, DAP and the hyper-reactivity to L-NNA remained elevated in the OVX rat.

It has been established that normal rat cerebral arteries were unresponsive to phenylephrine when the endothelium was intact (Thorin *et al.*, 1998a). Likewise, in human cerebral arteries, norepinephrine induced a limited contraction, as compared to serotonin (Bevan *et al.*, 1998; Thorin *et al.*, 1998b). By contrast, in the presence of NO synthase blockade, phenylephrine induced a contraction of normal rat cerebral arteries (Thorin *et al.*, 1998a). However, in the OVX rat, phenylephrine treatment promoted cerebral artery contraction in the absence of NO synthase inhibition, thereby supporting the premise that NO activity and/or synthesis was reduced. A strong predictive parameter to support this latter premise was the increased cerebrovascular sensitivity to SNP. It has been demonstrated that vascular smooth muscle sensitivity to NO negatively correlated to endogenous NO activity (Gilbert *et al.*, 2001).

It is difficult to reconcile the exaggerated contractile response to L-NNA and the concomitant increased sensitivity to SNP of arterial rings isolated from OVX rats. However, it is important to note that baseline tension was  $\text{Ca}^{2+}$  independent, and thus arterial segments had no active tone at rest before the

addition of L-NNA. Thus, the blockade of NO by L-NNA *per se* could not have contributed to the exaggerated contraction of cerebral arteries from OVX rats. The L-NNA-mediated inhibition of NO synthase may have provided the requisite environment for endothelium-derived contractile factors (EDCF) to induce a contraction (Nguyen *et al.*, 1999; Gilbert *et al.*, 2001). Conceivably, the augmented response to L-NNA may reflect a greater release of EDCF in the OVX rat. In this context, it is our contention that the increased vascular sensitivity in response to exogenous endothelin-1, high external  $\text{K}^+$ , as well as to phenylephrine (in the presence of L-NNA) observed 6-weeks post-OVX may in part reflect increased EDCF production. In addition, the enhanced vascular sensitivity to neurotransmitters and hormones may have contributed to the rise in systemic blood pressure. This view is strengthened by our recent report demonstrating that  $\text{ET}_A$  receptor inhibition prevented the rise in blood pressure associated with OVX (Mercier *et al.*, 2002). Endothelin-1 is a likely EDCF candidate considering its important regulatory role in human and rat pial arteries (Thorin *et al.*, 1998a, b).

In OVX rats, tamoxifen behaved primarily as a partial estrogen receptor agonist as increased body weight was normalized, and uterus atrophy was partially reversed. Moreover, tamoxifen therapy normalized the increased baseline tension of cerebral arteries, and abrogated the exaggerated phenylephrine-mediated contraction in the OVX rat. By contrast, tamoxifen treatment caused a modest decrease of SAP, was without effect on elevated DAP, and failed to inhibit either high  $\text{K}^+$  or L-NNA-mediated contractile response of the cerebral artery in the OVX rat. The two latter observations suggest that at least in the present study when tamoxifen was given 3 weeks post-OVX, the partial estrogen receptor agonist was unable to influence either vascular NO- or EDCF-dependent activity. At present, it is not possible to discern whether the beneficial effect of tamoxifen on cerebral vascular reactivity represented either a direct action or attributed to the modest secondary decrease of SAP.

In a recent study, Simon *et al.* (2002) reported that  $\geq 1$ -year tamoxifen therapy reduced carotid intima-media thickness in menopausal women, an effect highly significant with an impact on intima-media thickness of  $-70 \mu\text{m}$ . In the present study, the experimental model of ovariectomy was associated with elevated blood pressure, and in this regard significant cerebrovascular vessel remodelling may be prevalent (Hajdu *et al.*, 1991; Hajdu & Baumbach, 1994). Thus, it is tempting to speculate that changes in vessel structure may have contributed to the augmented resting tension and hyper-reactivity of cerebral arteries from OVX rats. Moreover, tamoxifen therapy normalized baseline tension and phenylephrine responsiveness of cerebral arteries from OVX rats, and partially reduced systolic blood pressure. It remains to be determined whether the beneficial action of tamoxifen may have occurred in part *via* the direct modulation of vessel remodelling.

Lacunar infarcts, which may account for a large proportion of strokes, are related to small vessel disease in the brain. Hypertension is a major risk factor in the pathogenesis of this form of vascular disease (Sacco *et al.*, 1997). The premise that HRT was an effective therapeutic approach in preventing stroke has been examined in several observational studies, and the conclusions remain equivocal (Tolbert & Oparil, 2001). Moreover, as previously mentioned, several recent clinical studies have demonstrated that HRT (estrogen and progesterone

regiment) was ineffective in the primary prevention of coronary artery disease in postmenopausal women (Hulley *et al.*, 1998; Rossouw *et al.*, 2002). It has been suggested that the coadministration of progesterone may limit the cardio-protective action of estrogen. Indeed, in surgically postmenopausal monkeys fed an atherogenic diet, coronary artery responsiveness to acetylcholine was improved in the estrogen-treated animals, whereas an amelioration was not observed in the estrogen–progesterone-treated group (Suparto *et al.*, 2003). In the present study, the administration of the partial estrogen receptor agonist tamoxifen in the ovariectomized rat markedly improved cerebral vascular reactivity. Thus, this study at least in part supports the concept that the activation

of an estrogen receptor-dependent pathway exerts a cardio-protective action. Lastly, although extrapolation from the experimental to the clinical setting is always subject to caution, the data of the present study suggest that SERMs may represent an alternative therapeutic approach in the treatment of cerebrovascular stroke in postmenopausal women.

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