

# Inducible nitric oxide synthase contributes to gender differences in ischemic brain injury

Eun-Mi Park<sup>1</sup>, Sunghee Cho<sup>2</sup>, Kelly A Frys, Sara B Glickstein, Ping Zhou, Josef Anrather, Margaret E Ross and Costantino Iadecola

Division of Neurobiology, Department of Neurology and Neuroscience, Weill Medical College of Cornell University, New York, New York, USA

**Estrogens have antiinflammatory actions and protect the brain from ischemic injury. Cerebral ischemia is accompanied by an inflammatory reaction that contributes to the tissue damage, an effect mediated in part by toxic amounts of nitric oxide (NO) produced by the inducible isoform of NO synthase (iNOS). Therefore, estrogens may protect the female brain by modulating postischemic iNOS expression. To test this hypothesis, we studied whether iNOS plays a role in the mechanisms of the reduced susceptibility to ischemic injury observed in female mice. The middle cerebral artery was occluded for 20 mins using an intraluminal filament in C57Bl/6 mice, and infarct volume was assessed 3 days later in cresyl violet-stained sections. Infarcts were 53% smaller in female mice than in males ( $P < 0.05$ ), a reduction abolished by ovariectomy (OVX) and reinstated by estrogen replacement. In normal female mice, postischemic iNOS mRNA was lower than in males ( $P < 0.05$ ). Ovariectomy increased iNOS mRNA after ischemia and estrogen replacement blocked this effect. Furthermore, the iNOS inhibitor aminoguanidine reduced infarct volume in male, but not in female, mice. Similarly, male iNOS-null mice had smaller infarcts than wild-type mice, but female iNOS nulls were not protected. Ovariectomy and OVX with estrogen replacement did not affect infarct volume in iNOS-null female mice. The findings suggest that the neuroprotection conferred by estrogens is, in part, related to attenuation of iNOS expression. Such attenuation could result from the potent antiinflammatory effects of estrogens that downregulate iNOS expression via transcriptional or posttranscriptional mechanisms.**

*Journal of Cerebral Blood Flow & Metabolism* (2006) 26, 392–401. doi:10.1038/sj.jcbfm.9600194; published online 27 July 2005

**Keywords:** aminoguanidine; ICAM-1; iNOS-null mice; NADPH oxidase; real-time PCR; stroke

## Introduction

There is considerable evidence that stroke is a sexually dimorphic disease. Thus, premenopausal women are relatively protected from cerebrovascular diseases, including ischemic stroke (Sudlow and Warlow, 1997). However, during menopause, the incidence of ischemic stroke and other cardiovascular diseases increases (Eaker *et al.*, 1993). These

epidemiological findings are supported by experimental studies in rodents, indicating that females are more resistant to cerebral ischemic injury (McCullough and Hurn, 2003; Wise *et al.*, 2001). Thus, the brain damage produced by focal or global cerebral ischemia is less marked in females than in male rodents (Alkayed *et al.*, 1998; Carswell *et al.*, 2004; Hall *et al.*, 1991; Jover *et al.*, 2002; Simpkins *et al.*, 1997). Such protection in females is abolished by ovariectomy (OVX) and reinstated by estrogen replacement, suggesting that estrogens are responsible for the protective effect (Dubal *et al.*, 1998; Rau *et al.*, 2003; Rusa *et al.*, 1999; Yang *et al.*, 2000).

The mechanisms by which estrogens protect from ischemic brain injury include both vascular effects leading to better preservation of cerebral blood flow (CBF) during ischemia (Alkayed *et al.*, 1998; McCullough *et al.*, 2001), and cytoprotective effects resulting in a greater tolerance of the brain to ischemia (Alkayed *et al.*, 2001; Simpkins *et al.*, 2005; Wang *et al.*, 1999). Estrogens also have remarkable antiinflammatory properties, the mechanisms of which have

Correspondence: Dr C Iadecola, Division of Neurobiology, Weill Medical College of Cornell University, 411 East 69th Street, Room KB410, New York, NY 10021, USA.

E-mail: coi2001@med.cornell.edu

<sup>1</sup>Current address: Department of Pharmacology, College of Medicine, Ewha Womans University, 911-1, Mok-6-Dong, Yangcheon-Gu, Seoul 158-710, Republic of Korea.

<sup>2</sup>Current address: Burke Medical Research Institute, 785 Mamaronck Ave, White Plains, NY 10605, USA.

This work was supported by NIH grants NS34179 and NS35806. CI is the recipient of a Javits Award from NIH/NINDS.

Received 10 May 2005; revised 14 June 2005; accepted 22 June 2005; published online 27 July 2005

not been elucidated (Baker *et al*, 2004; Bruce-Keller *et al*, 2000; Santizo *et al*, 2000; Vegeto *et al*, 2001, 2003).

Postischemic expression of the immunological or 'inducible' isoform of nitric oxide synthase (iNOS, NOS II) mediates some of the neurotoxic effects of inflammation (Iadecola *et al*, 2004). After focal cerebral ischemia, in rodents as in humans, iNOS is expressed in infiltrating inflammatory cells and in cerebral blood vessels within the ischemic territory (Forster *et al*, 1999; Galea *et al*, 1998; Hirabayashi *et al*, 2000; Iadecola *et al*, 1995b). Pharmacological inhibition of iNOS or downregulation of iNOS expression using antisense oligonucleotides reduces the infarct produced by middle cerebral artery (MCA) occlusion in rodents (Iadecola *et al*, 1995a; Parmentier *et al*, 1999; Parmentier-Batteur *et al*, 2001; Sugimoto and Iadecola, 2002), while iNOS-deficient mice are protected from focal cerebral ischemia (Iadecola *et al*, 1997; Loihl *et al*, 1999). The toxicity of iNOS has been attributed to the large amounts of nitric oxide (NO) produced by this enzyme, which results in oxidative stress, DNA damage, and inhibition of mitochondrial respiration (Keynes and Garthwaite, 2004). Considering the central role that iNOS plays in the pathogenic mechanisms of postischemic inflammation, it would be important to determine whether the reduced susceptibility to cerebral ischemia in females is related to suppression of the expression of this enzyme.

In the present study, we used a mouse model of focal cerebral ischemia-reperfusion to investigate whether iNOS contributes to the sexual dimorphism of ischemic brain injury. We found that postischemic iNOS expression is reduced in female mice, and that such reduction is abolished by OVX and reinstated by estrogen replacement. Furthermore, the iNOS inhibitor aminoguanidine (AG) did not reduce infarct volume in female mice. Similarly, female iNOS-null mice were not protected from ischemic injury. These findings suggest that estrogen-induced suppression of iNOS expression may contribute to the reduced susceptibility to ischemic injury in females.

## Materials and methods

### Mice

C57Bl/6 mice (age 2 to 3 months; weight 20 to 23 g) were obtained from Charles River (Wilmington, MA, USA). The inducible isoform of nitric oxide synthase-null mice (C57Bl/6 congenic) were obtained from an in-house colony (Cho *et al*, 2005b; Iadecola *et al*, 1997; Park *et al*, 2004).

### Transient Middle Cerebral Artery Occlusion

All procedures were approved by the Institutional Animal Care and Use Committee. As described in detail elsewhere (Cho *et al*, 2005a,b; Park *et al*, 2004), mice were anesthetized with isoflurane (1.5% to 2%). A fiber-optic probe was glued to the parietal bone (2 mm posterior and

5 mm lateral to bregma) and connected to a laser-Doppler flowmeter (Periflux System 5010, Perimed, Sweden) for continuous monitoring of CBF. For MCA occlusion, a heat-blunted surgical suture (6-0) was inserted into the exposed external carotid artery, advanced into the internal carotid artery, and wedged into the circle of Willis to obstruct the origin of the MCA. The filament was left in place for 20 mins and then withdrawn. Only animals that exhibited a reduction in CBF >85% during MCA occlusion and in which CBF recovered by >80% after 10 mins of reperfusion were included in the study. Rectal temperature was kept at  $37.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$  during surgery and in the recovery period, until animals regained consciousness. We used 20 mins of ischemia because longer durations did not allow the mice to survive for 72 h, the time at which infarct volume was assessed. The size of the ischemic lesion produced by 20 mins MCA occlusion was comparable to that observed in other studies of focal ischemia in mice (Dubal *et al*, 2001; Prass *et al*, 2003).

### Ovariectomy, Estrogen Replacement and Estrogen Assay

Female mice underwent aseptic bilateral surgical OVX via a dorsal incision under isoflurane anesthesia 2 weeks before MCA occlusion. One group of OVX mice received estrogen replacement, consisting of estrogen benzoate (Sigma, St Louis, MO, USA) ( $1 \mu\text{g}$  in  $100 \mu\text{L}$ , subcutaneously) (Qiu *et al*, 2003) for 6 days starting 3 days before MCAO. Vehicle-treated mice in the OVX group received sesame oil ( $100 \mu\text{L}$ , subcutaneously). Plasma estradiol levels were measured with a commercially available ELISA assay (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Uterine weight, an index of estrogen activity, was also measured.

### Infarct Volume Measurement and Aminoguanidine Treatment

Brains were removed, frozen, and sectioned (thickness:  $30 \mu\text{m}$ ) in a cryostat (Iadecola *et al*, 1997; Park *et al*, 2004). Brain sections were collected serially at  $600\text{-}\mu\text{m}$  intervals, and stained with cresyl violet. Infarct volume was determined using an image analyzer (MCID, Imaging Research Inc., St Catharines, Ontario, Canada). To eliminate the contribution of postischemic edema to the volume of injury, infarct volumes were corrected for swelling by comparing ischemic and nonischemic hemispheres as described (Cho *et al*, 2005a; Iadecola *et al*, 1997; Park *et al*, 2004). The iNOS inhibitor AG (Sigma) ( $100 \text{mg/kg}$  in saline, intraperitoneally) was administered twice a day (1000 and 1800 h) for 3 days, starting 10 mins after reperfusion (Park *et al*, 2004). Vehicle (saline)-injected mice served as controls.

### Quantitative 'Real-Time' PCR

Procedures for real-time PCR have been described previously (Park *et al*, 2004). Briefly, mice in which ischemia

was induced ( $n = 4/\text{group}$ ) were killed 6, 24, and 72 h after reperfusion. Sham-operated mice served as controls ( $n = 4/\text{group}$ ). Total RNA was prepared from the ischemic and contralateral hemispheres using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Quantitative determination of gene expression levels, employing a two-step cycling protocol, was performed on a Chromo 4 detector (Peltier Thermal Cycler) (MJ Research, Waltam, MA, USA). Primers for iNOS (forward, 5'-CAGCTGGGCTG TACAAACCTT-3', and reverse, 5'-CATTGGAAGTCAAGC GTTTCG-3'), ICAM (forward, 5'-GCCTTGGTAGAGGTGA CTGAG-3', and reverse, 5'-GACCGGAGCTGAAAAGTTG TA-3'), and gp91<sup>phox</sup> (5'-CCAACCTGGGATAACGAGTTCA-3', and reverse, 5'-GAGAGTTTCAGCCAAGGCTTC-3') were purchased from Invitrogen Life Technologies. In all, 2  $\mu\text{L}$  of diluted cDNA (1:10) was amplified by Platinum SYBR green qPCR supermix UDG (Invitrogen Life Technologies). The reactions were incubated at 50°C for 2 mins and then at 95°C for 10 mins. A PCR cycling protocol consisting of 15 secs at 95°C and 1 min at 60°C for 45 cycles was required for quantification, and relative expression levels were calculated (Livak and Schmittgen, 2001). Quantities of all targets in the test samples were normalized to the mouse HPRT house-keeping gene, and values were normalized to those of sham-operated mice.

### Immunocytochemistry

Immunocytochemical procedures were identical to those described previously (Cho *et al*, 2005b; Iadecola *et al*, 1996). Sections (7  $\mu\text{m}$ ) from formalin-fixed, paraffin-embedded brains were incubated overnight (4°C) with an iNOS polyclonal antibody (Upstate Biotechnology Incorporated, Lake Placid, NY, USA; dilution 1:5000), or with antibodies to the peroxynitrite marker 3-nitrotyrosine (NT) (Upstate Biotechnology Incorporated; dilution 1:2000) (Cho *et al*, 2005b). In certain conditions associated with inflammation, tyrosine nitration can also be promoted by nitrite (Eiserich *et al*, 1998). However, in the absence of florid inflammation, this is unlikely to be a relevant factor affecting the specificity of NT as a marker of peroxynitrite. Sections were washed and incubated with the secondary antibody (Vector) for 30 mins. The immunocomplex was visualized using the ABC complex method (Vectastain Elite Kit, Vector). The specificity of the immunolabel was previously tested by preadsorption of the antigen and by removing the primary antibody (Forster *et al*, 1999).

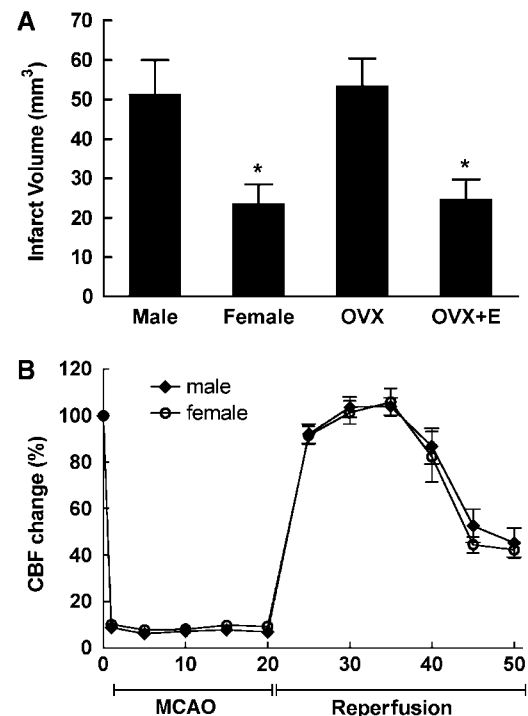
### Statistical Analysis

Data are expressed as mean  $\pm$  s.e.m. Two-group comparisons were statistically evaluated by the Student's *t*-test. Multiple comparisons were evaluated by the analysis of variance and Fisher's protected least significant difference (PLSD) test. Differences were considered significant at  $P < 0.05$ .

## Results

### Sex Differences in Ischemic Brain Injury are Abolished by Ovariectomy and Reestablished by Estrogen Replacement

Middle cerebral artery occlusion produced well-defined infarcts involving both the cortex and the striatum. In agreement with reports by others (e.g., Alkayed *et al*, 1998), infarct volume was larger in male than in female mice (Figure 1A). The changes in CBF produced by MCA occlusion and reperfusion did not differ in males and females (Figure 1B). In female mice, OVX reduced estradiol levels and uterine weights, effects that were counteracted by estrogen replacement (Table 1). The reduction in estradiol levels in OVX did not reach statistical significance due to variability associated with estrogen levels in randomly cycling females (Table 1). Ovariectomy increased infarct volume to levels comparable to those observed in male mice (Figure 1A), while estrogen replacement decreased infarct volume to the level seen in intact females (Figure 1A). These findings establish that sex differences in stroke volume are present in our model, and that they are abolished by OVX and reinstated by estrogen replacement.



**Figure 1** Sex differences in ischemic brain injury produced by transient occlusion of the MCA. **(A)** Infarct volumes in male ( $n = 10$ ), female ( $n = 12$ ), ovariectomized (OVX) ( $n = 9$ ) and OVX + estrogen (E) ( $n = 7$ ).  $*P < 0.05$  from male and OVX (analysis of variance and Fisher's PLSD test). **(B)** Cerebral blood flow changes during ischemia and reperfusion in male ( $n = 11$ ) and female mice ( $n = 8$ ).

**Table 1** Effect of ovariectomy and estrogen replacement on plasma estradiol concentration and uterine weight in female WT and iNOS-null mice

<i>C57Bl/6</i> mice	Intact (n = 6)	OVX (n = 8)	OVX+E (n = 5)
Serum estradiol (pg/mL)	5.6 ± 0.8	2.7 ± 1.1	17.0 ± 2.3 <sup>#</sup>
Uterine weight (mg)	31.5 ± 3.1	14.3 ± 1.0*	87.3 ± 5.0 <sup>#</sup>
<i>iNOS</i> <sup>-/-</sup> mice	Intact (n = 5)	OVX (n = 5)	OVX+E (n = 5)
Serum estradiol (pg/mL)	5.3 ± 1.4	2.9 ± 0.4	14.8 ± 2.1 <sup>#</sup>
Uterine weight (mg)	46.0 ± 7.8	15.4 ± 1.3*	90.7 ± 6.7 <sup>#</sup>

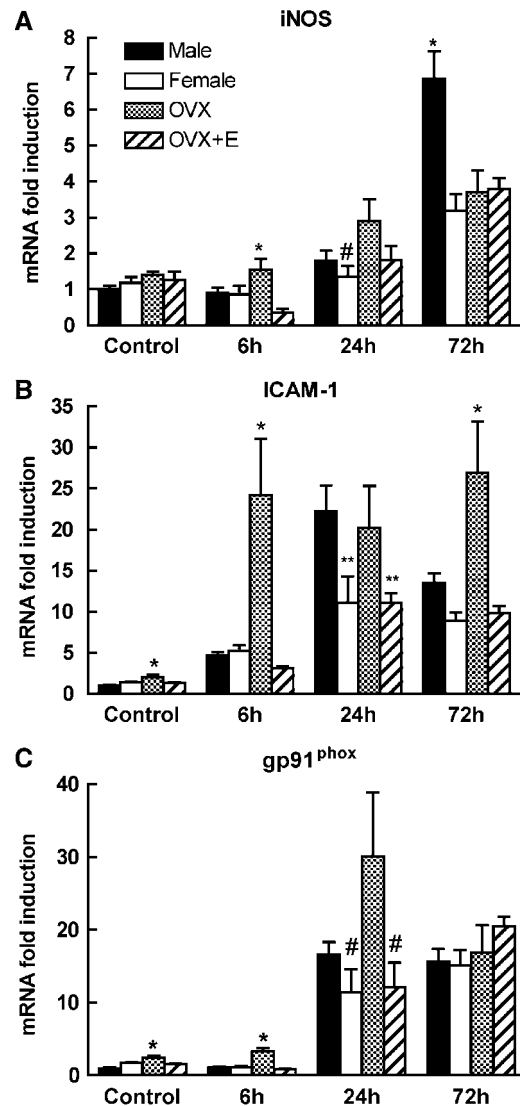
OVX, ovariectomy; OVX+E, OVX with estrogen replacement.  
\**P* < 0.05 from intact; <sup>#</sup>*P* < 0.05 from intact and OVX (analysis of variance).

**Postischemic Inducible Isoform of Nitric Oxide Synthase mRNA Expression is Attenuated in Female Mice**

We next examined whether changes in postischemic iNOS expression could contribute to sex differences in the outcome of cerebral ischemia. In male mice, iNOS mRNA was elevated at 24 and 72 h after MCA occlusion (Figure 2A) (*P* < 0.05 from control). However, in female mice, iNOS expression was markedly lower (Figure 2A) (−53% at 72 h; *P* < 0.05 from males). Ovariectomy enhanced iNOS mRNA at 6 and 24 h after ischemia (*P* < 0.05), but not at 72 h (Figure 2A) (*P* > 0.05). The iNOS upregulation induced by OVX was reversed by estrogen replacement (Figure 2A) (*P* > 0.05 from females). To determine whether other inflammatory genes were also influenced by gender, we investigated the postischemic mRNA expression of the adhesion molecule ICAM-1 and the NADPH oxidase subunit gp91<sup>phox</sup>. ICAM-1 mRNA expression was increased after ischemia reaching a maximum at 24 h (Figure 2B) (*P* < 0.05). The elevation was much greater in male than in normal female mice (Figure 2B) (*P* < 0.05). ICAM-1 expression was markedly enhanced by OVX, including the time point before ischemia, but this enhancement was attenuated to levels observed in intact females by estrogen replacement (Figure 2B). gp91<sup>phox</sup> mRNA upregulation was only slightly reduced in female mice at 24 h, and did not differ between male and females at 72 h (Figure 2C) (*P* > 0.05). Ovariectomy enhanced gp91<sup>phox</sup> expression (Figure 2C). The effect was observed in controls, and 6 h after ischemia, and was counteracted by estrogen replacement (Figure 2C).

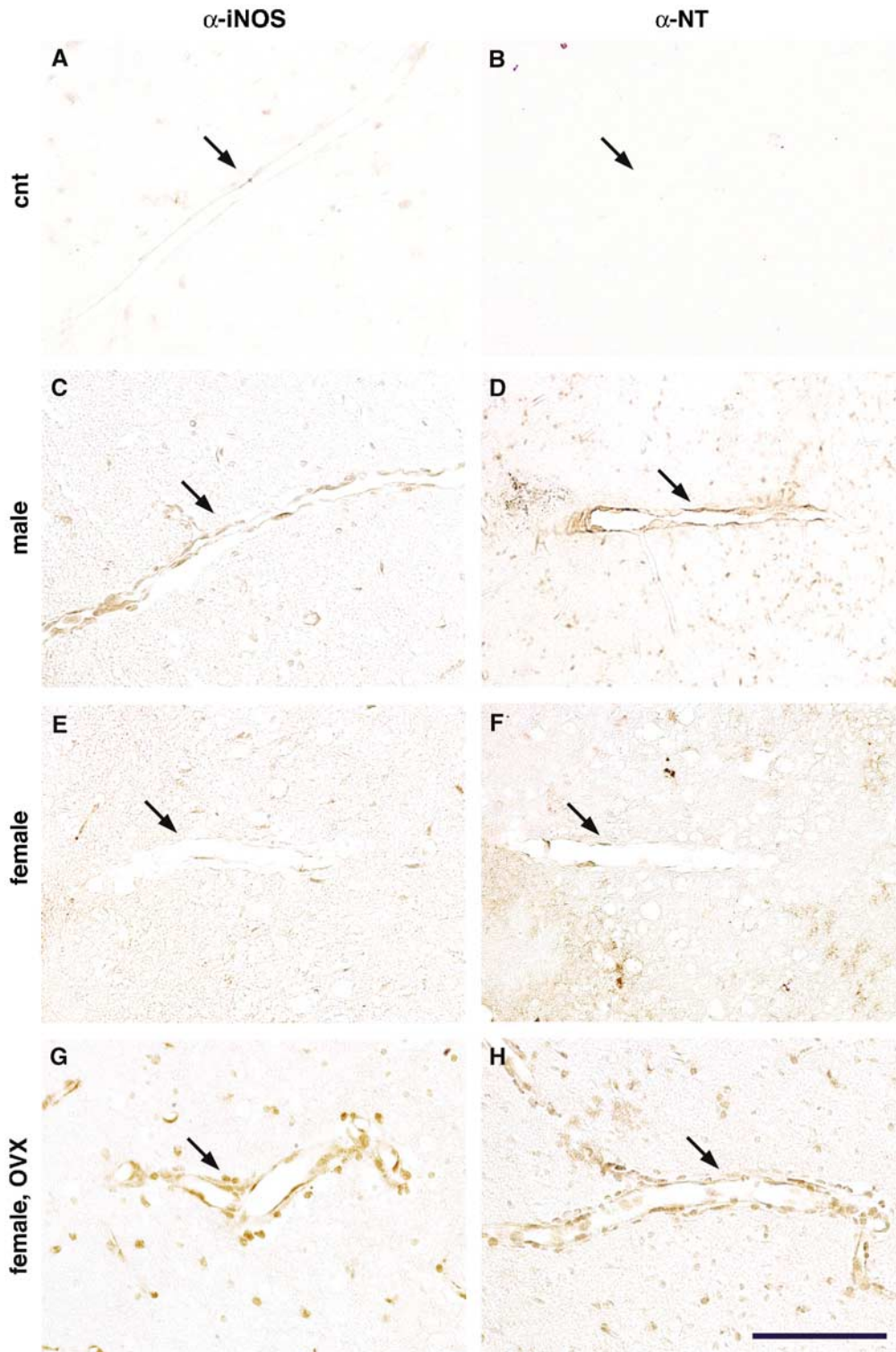
**Postischemic Inducible Isoform of Nitric Oxide Synthase Immunoreactivity is Attenuated in Female Mice**

We used immunocytochemistry to determine whether the attenuation in iNOS mRNA expression was associated with a reduction in iNOS protein



**Figure 2** Time course of the expression of iNOS (A), ICAM-1 (B) and the NADPH oxidase subunit gp91<sup>phox</sup> (C) mRNA in brain after transient focal cerebral ischemia in male, female, OVX, and OVX + E mice. mRNA was assessed by the quantitative real-time polymerase chain reaction (*n* = 4/group). The control group consisted of sham-operated mice. \**P* < 0.05 compared with the other groups at the same time point, <sup>#</sup>*P* < 0.05 compared with OVX, \*\**P* < 0.05 compared with male (analysis of variance and Fisher's PLSD test).

72 h after ischemia–reperfusion. In male mice (*n* = 3), iNOS immunoreactivity was observed in the wall of cerebral blood vessels and in inflammatory cells in the ischemic territory, as described previously (Iadecola *et al*, 1995b, 1996) (Figure 3A). Immunoreactivity for NT, a marker of the NO reaction product peroxynitrite, was also observed in inflammatory cells and cerebral blood vessels (Figure 3B). The inducible isoform of nitric oxide synthase and NT immunoreactivity were markedly lower in the brains of female mice (*n* = 3) (Figures 3C and 3D), but the expression was enhanced by OVX (*n* = 3) (Figures 3E and 3F).



**Figure 3** Expression of iNOS or NT immunoreactivity in the postischemic brain of male, female, and OVX mice, 72 h after MCA occlusion. (A, B) iNOS or NT immunoreactivity was not observed in the nonischemic brain. (C, D) In male mice, there is iNOS and NT immunoreactivity in blood vessels (arrow) and infiltrating inflammatory cells throughout the infarct. (E, F) iNOS and NT immunoreactivity are markedly attenuated in intact female mice. (G, H) Ovariectomy increases iNOS and NT immunoreactivity in the postischemic brain. Calibration bar = 100  $\mu$ m.

### Pharmacological Inhibition or Genetic Deletion of Inducible Isoform of Nitric Oxide Synthase does not Confer Protection in Female Mice

The observation that iNOS expression is attenuated in female mice suggests that the component of ischemic damage attributable to iNOS is not present in females. To test this hypothesis, we examined the protective effect of the iNOS inhibitor AG and iNOS deletion in male and female mice. Aminoguanidine attenuated infarct volume in male (−55%), but not in female, mice (Figure 4A). Similarly, infarct volume was reduced in male (−45%), but not in female, iNOS-null mice (Figure 4B), an effect independent of changes in postischemic CBF (Figure 4C). Therefore, the protective effects of inhibition or genetic deletion of iNOS are not observed in female mice.

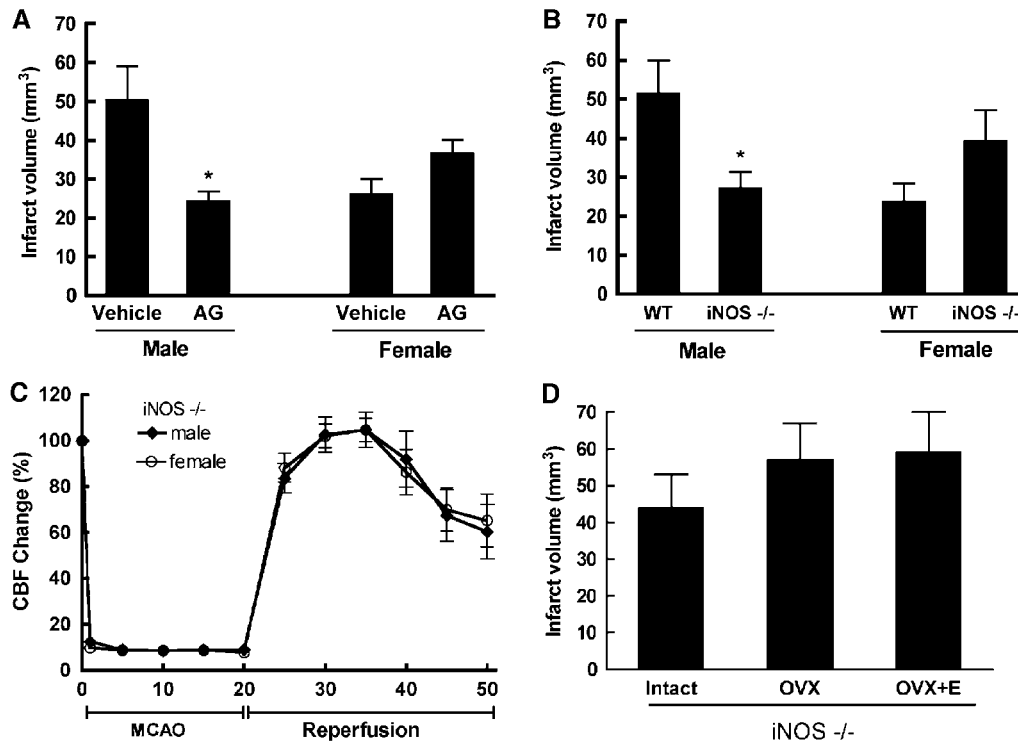
### Estrogens do not Confer Protection in Female Inducible Isoform of Nitric Oxide Synthase-Null Mice

To determine whether the protection exerted by estrogens requires iNOS for its expression, we investigated the effect of OVX and estrogen replacement in iNOS-null mice. In iNOS nulls, estradiol levels and uterine weights were reduced by OVX

and reestablished by estrogen replacement (Table 1). Ovariectomy produced a small increase in stroke volume in iNOS-null mice, which did not reach statistical significance in this small group (Figure 4D) ( $P > 0.05$ ). Unlike wild-type (WT) mice (Figure 1A), estrogen replacement after OVX did not reduce injury volume in iNOS nulls (Figure 4D) ( $P > 0.05$ ).

## Discussion

We have shown that female mice are relatively protected from focal cerebral ischemia and that such protection is associated with a reduction in post-ischemic iNOS expression compared with male mice subjected to a comparable ischemic insult. Ovariectomy abolished the reduction in ischemic injury observed in female mice and increased iNOS expression at 6 and 24 h, while estrogen replacement attenuated both iNOS expression and ischemic injury. Furthermore, treatment with the iNOS inhibitor AG reduced ischemic injury in male, but not in female, mice. Similarly, the reduction in ischemic injury observed in male iNOS-null mice was not observed in female nulls, a finding reported previously by others (Loihs *et al*, 1999). These observations, collectively, suggest that



**Figure 4** Sex differences in the effect of iNOS inhibition on ischemic injury. (A) Effects of the iNOS inhibitor AG (male,  $n = 8$ ; female,  $n = 6$ ) on infarct volume in WT mice. Vehicle-treated groups (male,  $n = 8$ ; female,  $n = 7$ ) received normal saline (100  $\mu$ L; intraperitoneal) twice/day. \* $P < 0.05$  from vehicle-treated male mice (Student's  $t$ -test). (B) Effects of iNOS gene deletion on infarct volume in male ( $n = 7$ ) and female ( $n = 9$ ) mice; \* $P < 0.05$  from WT male mice ( $t$ -test). No statistical difference was observed between female WT and iNOS-null mice ( $P > 0.05$ ). (C) Cerebral blood flow changes during ischemia and reperfusion in male ( $n = 7$ ) and female iNOS-null mice ( $n = 9$ ). (D) Effect of OVX and OVX + estrogen in iNOS-null female mice ( $P > 0.05$ ; analysis of variance;  $n = 5$ /group).

the component of ischemic brain injury attributable to iNOS is lacking in female mice, and support the hypothesis that reduced iNOS expression contributes to the protection observed in females.

Smaller infarcts tend to have a less pronounced inflammatory response (Akopov *et al*, 1996; Kochanek and Hallenbeck, 1992). However, the reduction in iNOS mRNA expression is unlikely to be the consequence of the smaller infarcts in female mice because our data show that the magnitude of iNOS induction at 72 h is not proportional to the final size of the infarct. For example, OVX mice had a level of iNOS mRNA at 72 h similar to that observed in intact females or estrogen-treated OVX mice, but their infarct volume was twice as large. Consistent with a causal link between reduced iNOS expression and reduced infarct size, the iNOS inhibitor AG was not effective in female mice and female iNOS-null mice were not protected from cerebral ischemia. These observations support the conclusion that the reduction in iNOS expression in females is causally related to the smaller stroke rather than a consequence of reduced tissue injury. The lack of reduction in infarct volume in female iNOS-null mice or female mice treated with AG is unlikely to result from the fact that the infarct was already maximally reduced. This is because it has been previously shown that infarct volume in female mice can be attenuated, for example, by overexpression of the free radical scavenging enzyme superoxide dismutase (SOD) (Sampei *et al*, 2000b). In the present study, estrogen replacement in OVX females produced increases in plasma estradiol that were higher than those observed in intact females. However, the differences in the outcome of cerebral ischemia cannot be attributed to estrogen levels because plasma levels did not differ between WT and iNOS-null mice, and were in the range reported by others (Dubal *et al*, 2001; Horsburgh *et al*, 2002).

The sex differences in the susceptibility to focal cerebral ischemia have been attributed to estrogens, hormones that can modulate cerebral ischemic injury through a wide variety of actions (McCullough and Hurn, 2003; Wise *et al*, 2001). Some of these actions, including effects on gene expression and intracellular signaling, are mediated by estrogen receptors (ER), while other actions, such as the antioxidant properties, are receptor independent (Maggi *et al*, 2004). Estrogens have long been known to attenuate the expression of proinflammatory factors (Behl, 2002; Wise *et al*, 2001). In brain, estrogens reduce the expression of inflammatory genes, including iNOS, in activated microglial cells (Baker *et al*, 2004; Drew and Chavis, 2000). Although the protective effect resulting from suppression of iNOS by estrogens is well documented in cell culture and in models of brain inflammation (Drew and Chavis, 2000; Vegeto *et al*, 2003), the role of iNOS in the protection afforded by estrogens in ischemic brain injury is less well defined. The findings of the present study show for the first time

that suppression of iNOS is an important factor in the neuroprotective effect of estrogens in models of focal ischemia.

In addition to iNOS, the postischemic expression of other inflammation-related genes was reduced in female mice. For example, the adhesion molecule ICAM-1 was attenuated in female mice, an effect abolished by OVX and reinstated by estrogen replacement. Considering the deleterious role that ICAM-1 plays in the mechanisms of ischemic brain injury (Bowes *et al*, 1993; Connolly *et al*, 1996), it is conceivable that the downregulation of this adhesion molecule also contributes to the reduced susceptibility to cerebral ischemia in female mice. Suppression of postischemic ICAM-1 expression may underlie the reduced leukocyte adhesion reported after cerebral ischemia in female mice (Santizo and Pelligrino, 1999; Santizo *et al*, 2000). Similarly, the postischemic expression of the NADPH oxidase subunit gp91<sup>phox</sup> was reduced in female mice. NADPH oxidase is upregulated after cerebral ischemia and contributes to ischemic injury, presumably by producing free radicals (Kusaka *et al*, 2004; Walder *et al*, 1997). Therefore, the attenuation in gp91<sup>phox</sup> expression may also play a role in the reduction in ischemic injury in females. The observation that gp91<sup>phox</sup> expression is enhanced by OVX and attenuated by estrogen replacement attests to the estrogen dependence of the effect. Therefore, in addition to iNOS, other genes are also likely to be involved in the sexual dimorphism of ischemic brain injury.

The involvement of genes other than iNOS in the deleterious effects of OVX on ischemic damage is also indicated by the fact that OVX enlarged the infarct in iNOS-null mice. However, the exacerbation of the damage in iNOS nulls (+29%) was small compared with that observed in WT mice (+100%), and did not reach statistical significance. However, the fact that estrogen replacement did not attenuate the lesion suggests that iNOS is required for the neuroprotective effect of estrogens. This observation is in line with other data supporting the view that iNOS is not always deleterious to the postischemic brain. Thus, iNOS is an absolute requirement for the neuroprotection exerted by ischemic preconditioning or by the preconditioning induced by the proinflammatory agent lipopolysaccharide (Cho *et al*, 2005b). Furthermore, iNOS is required for the protective effect of PARP inhibition (Park *et al*, 2004). Therefore, in addition to its deleterious role in ischemic brain injury, iNOS has also beneficial effects related to ischemic preconditioning and to the neuroprotection conferred by estrogens and PARP inhibitors.

The mechanisms by which estrogens downregulate the expression of iNOS and other inflammatory genes remain unclear, because some of these genes may lack an estrogen response element (Baker *et al*, 2004). Therefore, activation of ER is thought to induce intracellular signaling that ultimately leads

to activation of the transcription factors AP1 and NF- $\kappa$ B, which, in turn, induce the expression of iNOS and other inflammatory genes (Baker *et al*, 2004). Although estrogens have been shown to reduce NF- $\kappa$ B activation in focal cerebral ischemia (Wen *et al*, 2004) and in astrocytic cultures treated with lipopolysaccharide (Dodel *et al*, 1999), in microglial cultures estrogens reduce iNOS expression without attenuating NF- $\kappa$ B activation (Bruce-Keller *et al*, 2000). However, ER signaling leads to activation of MAP kinase, which has been linked to the anti-inflammatory effect of estrogens (Maggi *et al*, 2004). Furthermore, ER signaling also leads to activation of the cAMP/PKA/CREB and PI3K/AKT protective pathways (Maggi *et al*, 2004). Therefore, it remains unclear whether the antiinflammatory effects of estrogens are mediated transcriptionally or posttranscriptionally. In addition, there is no consensus about the ER subtype ( $\alpha$  or  $\beta$ ) involved in the antiinflammatory and neuroprotective effects of estrogens (Baker *et al*, 2004; Carswell *et al*, 2004; Dubal *et al*, 2001; Sampei *et al*, 2000a; Vegeto *et al*, 2003). Therefore, further investigations are needed to define the receptor interactions and signaling through which estrogens regulate postischemic inflammation.

The observation that females are not susceptible to the beneficial effects of iNOS inhibition supports the view that a particular neuroprotective strategy cannot be assumed to be effective in both sexes. For example, infarct volume is reduced in male, but not in female, nNOS-null mice (Sampei *et al*, 2000b). Furthermore, infarct volume is markedly reduced in male mice lacking the DNA repair enzyme PARP, but it is exacerbated in female PARP-null mice (McCullough *et al*, 2005). Other neuroprotective strategies, such as overexpression of the free radical scavenger SOD, are effective in both sexes (Sampei *et al*, 2000b). The sexual dimorphism of the protective effect of iNOS inhibition has to be taken into consideration in the development of pharmacological treatments for human stroke.

In conclusion, we have shown that postischemic iNOS expression is lower in the brains of female mice. The iNOS expression is enhanced by OVX and reinstated by estrogen replacement. Furthermore, female mice are not susceptible to the protective effects of the iNOS inhibitor AG, while female iNOS-null mice are not protected from ischemic injury. These findings suggest that modulation of iNOS expression by estrogens is one of the factors mediating the resistance to cerebral ischemia in females, and provide additional evidence that neuroprotective strategies cannot be assumed to be effective in both sexes.

## Acknowledgements

We thank Dr Carrie Drake for comments on an earlier version of the manuscript.

## References

- Akopov SE, Simonian NA, Grigorian GS (1996) Dynamics of polymorphonuclear accumulation in acute cerebral infarction and their correlation with brain tissue damage. *Stroke* 27:1739–43
- Alkayed NJ, Goto S, Sugo N, Joh HD, Klaus J, Crain BJ, Bernard O, Traystman RJ, Hurn PD (2001) Estrogen and Bcl-2: gene induction and effect of transgene in experimental stroke. *J Neurosci* 21:7543–50
- Alkayed NJ, Harukuni I, Kimes , London ED, Traystman RJ, Hurn PD (1998) Gender-linked brain injury in experimental stroke. *Stroke* 29:159–65
- Baker AE, Brautigam VM, Watters JJ (2004) Estrogen modulates microglial inflammatory mediator production via interactions with estrogen receptor beta. *Endocrinology* 145:5021–32
- Behl C (2002) Oestrogen as a neuroprotective hormone. *Nat Rev Neurosci* 3:433–42
- Bowes MP, Zivin JA, Rothlein R (1993) Monoclonal antibody to the ICAM-1 adhesion site reduces neurological damage in a rabbit cerebral embolism stroke model. *Exp Neurol* 119:215–9
- Bruce-Keller AJ, Keeling JL, Keller JN, Huang FF, Camondola S, Mattson MP (2000) Antiinflammatory effects of estrogen on microglial activation. *Endocrinology* 141:3646–56
- Carswell HV, Macrae IM, Gallagher L, Harrop E, Horsburgh KJ (2004) Neuroprotection by a selective estrogen receptor beta agonist in a mouse model of global ischemia. *Am J Physiol Heart Circ Physiol* 287:H1501–4
- Cho S, Park EM, Febbraio M, Anrather J, Park L, Racchumi G, Silverstein RL, Iadecola C (2005a) The class B scavenger receptor CD36 mediates free radical production and tissue injury in cerebral ischemia. *J Neurosci* 25:2504–12
- Cho S, Park EM, Zhou P, Frys K, Ross ME, Iadecola C (2005b) Obligatory role of inducible nitric oxide synthase in ischemic preconditioning. *J Cereb Blood Flow Metab* 25:493–501
- Connolly ES, Jr, Winfree CJ, Springer TA, Naka Y, Liao H, Yan SD, Stern DM, Solomon RA, Gutierrez-Ramos JC, Pinsky DJ (1996) Cerebral protection in homozygous null ICAM-1 mice after middle cerebral artery occlusion. Role of neutrophil adhesion in the pathogenesis of stroke. *J Clin Invest* 97:209–16
- Dodel RC, Du Y, Bales KR, Gao F, Paul SM (1999) Sodium salicylate and 17 $\beta$ -estradiol attenuate nuclear transcription factor NF- $\kappa$ B translocation in cultured rat astroglial cultures following exposure to amyloid A beta(1–40) and lipopolysaccharides. *J Neurochem* 73:1453–60
- Drew PD, Chavis JA (2000) Female Sex steroids: effects upon microglial cell activation. *J Neuroimmunol* 111:77–85
- Dubal DB, Kashon ML, Pettigrew LC, Ren JM, Finklestein SP, Rau SW, Wise PM (1998) Estradiol protects against ischemic injury. *J Cereb Blood Flow Metab* 18:1253–8
- Dubal DB, Zhu H, Yu J, Rau SW, Shughrue PJ, Merchenthaler I, Kindy MS, Wise PM (2001) Estrogen receptor alpha, not beta, is a critical link in estradiol-mediated protection against brain injury. *Proc Natl Acad Sci USA* 98:1952–7
- Eaker ED, Chesebro JH, Sacks FM, Wenger NK, Whisnant JP, Winston M (1993) Cardiovascular disease in women. *Circulation* 88:1999–2009

- Eiserich JP, Hristova M, Cross CE, Jones AD, Freeman BA, Halliwell B, van der Vliet A (1998) Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 391:393–7
- Forster C, Clark HB, Ross ME, Iadecola C (1999) Inducible nitric oxide synthase expression in human cerebral infarcts. *Acta Neuropathol (Berl)* 97:215–20
- Galea E, Golanov EV, Feinstein DL, Kobylarz KA, Glickstein SB, Reis DJ (1998) Cerebellar stimulation reduces inducible nitric oxide synthase expression and protects brain from ischemia. *Am J Physiol* 274: H2035–45
- Hall ED, Pazara KE, Linseman KL (1991) Sex differences in postischemic neuronal necrosis in gerbils. *J Cereb Blood Flow Metab* 11:292–8
- Hirabayashi H, Takizawa S, Fukuyama N, Nakazawa H, Shinohara Y (2000) Nitrotyrosine generation via inducible nitric oxide synthase in vascular wall in focal ischemia-reperfusion. *Brain Res* 852:319–25
- Horsburgh K, Macrae IM, Carswell H (2002) Estrogen is neuroprotective via an apolipoprotein E-dependent mechanism in a mouse model of global ischemia. *J Cereb Blood Flow Metab* 22:1189–95
- Iadecola C, Cho S, Feuerstein GZ, Hallenbeck J (2004) Cerebral ischemia and inflammation. In: *Stroke: pathophysiology, diagnosis, and management* (Moore JP, Choi D, Grotta JC et al, eds), New York: Churchill Livingstone, 883–94
- Iadecola C, Zhang F, Casey R, Clark HB, Ross ME (1996) Inducible nitric oxide synthase gene expression in vascular cells after transient focal cerebral ischemia. *Stroke* 27:1373–80
- Iadecola C, Zhang F, Casey R, Nagayama M, Ross ME (1997) Delayed reduction in ischemic brain injury and neurological deficits in mice lacking the inducible nitric oxide synthase gene. *J Neurosci* 17:9157–64
- Iadecola C, Zhang F, Xu X (1995a) Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage. *Am J Physiol* 268:R286–92
- Iadecola C, Zhang F, Xu X, Casey R, Ross ME (1995b) Inducible nitric oxide synthase gene expression in brain following cerebral ischemia. *J Cereb Blood Flow Metab* 15:378–84
- Jover T, Tanaka H, Calderone A, Oguro K, Bennett MV, Etgen AM, Zukin RS (2002) Estrogen protects against global ischemia-induced neuronal death and prevents activation of apoptotic cascades in the hippocampal CA1. *J Neurosci* 22:2115–24
- Keynes RG, Garthwaite J (2004) Nitric oxide and its role in ischaemic brain injury. *Curr Mol Med* 4:179–91
- Kochanek PM, Hallenbeck JM (1992) Polymorphonuclear leukocytes and monocyte/macrophages in the pathogenesis of cerebral ischemia and stroke. *Stroke* 23: 1367–79
- Kusaka I, Kusaka G, Zhou C, Ishikawa M, Nanda A, Granger DN, Zhang JH, Tang J (2004) Role of AT1 receptors and NAD(P)H oxidase in diabetes-aggravated ischemic brain injury. *Am J Physiol Heart Circ Physiol* 286:H2442–51
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-ΔΔC<sub>T</sub></sup> method. *Methods* 25:402–8
- Loihl AK, Asensio V, Campbell IL, Murphy S (1999) Expression of nitric oxide synthase (NOS)-2 following permanent focal ischemia and the role of nitric oxide in infarct generation in male, female and NOS-2 gene-deficient mice. *Brain Res* 830:155–64
- Maggi A, Ciana P, Belcredito S, Vegeto E (2004) Estrogens in the nervous system: mechanisms and nonreproductive functions. *Annu Rev Physiol* 66:291–313
- McCullough LD, Alkayed NJ, Traystman RJ, Williams MJ, Hurn PD (2001) Postischemic estrogen reduces hypoperfusion and secondary ischemia after experimental stroke. *Stroke* 32:796–802
- McCullough LD, Hurn PD (2003) Estrogen and ischemic neuroprotection: an integrated view. *Trends Endocrinol Metab* 14:228–35
- McCullough LD, Zeng Z, Blizzard KK, Debchoudhury I, Hurn PD (2005) Ischemic nitric oxide and poly(ADP-ribose) polymerase-1 in cerebral ischemia: male toxicity, female protection. *J Cereb Blood Flow Metab* 25:502–12
- Park E-M, Cho S, Frys K, Racchumi G, Zhou P, Anrather J, Iadecola C (2004) Interaction between inducible nitric oxide synthase and poly(ADP-ribose) polymerase in focal ischemic brain injury. *Stroke* 35:2896–901
- Parmentier S, Bohme GA, Lerouet D, Damour D, Stutzmann JM, Margail I, Plotkine M (1999) Selective inhibition of inducible nitric oxide synthase prevents ischaemic brain injury. *Br J Pharmacol* 127: 546–52
- Parmentier-Batteur S, Bohme GA, Lerouet D, Zhou-Ding L, Beray V, Margail I, Plotkine M (2001) Antisense oligodeoxynucleotide to inducible nitric oxide synthase protects against transient focal cerebral ischemia-induced brain injury. *J Cereb Blood Flow Metab* 21:15–21
- Prass K, Scharff A, Ruscher K, Lowl D, Muselmann C, Victorov I, Kapinya K, Dirnagl U, Meisel A (2003) Hypoxia-induced stroke tolerance in the mouse is mediated by erythropoietin. *Stroke* 34:1981–6
- Qiu J, Bosch MA, Tobias SC, Grandy DK, Scanlan TS, Ronnekleiv OK, Kelly MJ (2003) Rapid signaling of estrogen in hypothalamic neurons involves a novel G-protein-coupled estrogen receptor that activates protein kinase C. *J Neurosci* 23:9529–40
- Rau SW, Dubal DB, Bottner M, Gerhold LM, Wise PM (2003) Estradiol attenuates programmed cell death after stroke-like injury. *J Neurosci* 23:11420–6
- Rusa R, Alkayed NJ, Crain BJ, Traystman RJ, Kimes , London ED, Klaus JA, Hurn PD (1999) 17β-estradiol reduces stroke injury in estrogen-deficient female animals. *Stroke* 30:1665–70
- Sampei K, Goto S, Alkayed NJ, Crain BJ, Korach KS, Traystman RJ, Demas GE, Nelson RJ, Hurn PD (2000a) Stroke in estrogen receptor-α-deficient mice. *Stroke* 31:738–43
- Sampei K, Mandir AS, Asano Y, Wong PC, Traystman RJ, Dawson VL, Dawson TM, Hurn PD (2000b) Stroke outcome in double-mutant antioxidant transgenic mice. *Stroke* 31:2685–91
- Santizo R, Pelligrino DA (1999) Estrogen reduces leukocyte adhesion in the cerebral circulation of female rats. *J Cereb Blood Flow Metab* 19:1061–5
- Santizo RA, Anderson S, Ye S, Koenig HM, Pelligrino DA (2000) Effects of estrogen on leukocyte adhesion after transient forebrain ischemia. *Stroke* 31:2231–5
- Simpkins JW, Rajakumar G, Zhang YQ, Simpkins CE, Greenwald D, Yu CJ, Bodor N, Day AL (1997) Estrogens may reduce mortality and ischemic damage caused by middle cerebral artery occlusion in the female rat. *J Neurosurg* 87:724–30
- Simpkins JW, Wang J, Wang X, Perez E, Prokai L, Dykens JA (2005) Mitochondria play a central role in

- estrogen-induced neuroprotection. *Curr Drug Targets CNS Neurol Disord* 4:69–83
- Sudlow CL, Warlow CP (1997) Comparable studies of the incidence of stroke and its pathological types: results from an international collaboration. International Stroke IncidenceCollaboration. *Stroke* 28:491–9
- Sugimoto K, Iadecola C (2002) Effects of aminoguanidine on cerebral ischemia in mice: comparison between mice with and without inducible nitric oxide synthase gene. *Neurosci Lett* 331:25–8
- Vegeto E, Belcredito S, Etteri S, Ghisletti S, Brusadelli A, Meda C, Krust A, Dupont S, Ciana P, Chambon P, Maggi A (2003) Estrogen receptor-alpha mediates the brain antiinflammatory activity of estradiol. *Proc Natl Acad Sci USA* 100:9614–9
- Vegeto E, Bonincontro C, Pollio G, Sala A, Viappiani S, Nardi F, Brusadelli A, Viviani B, Ciana P, Maggi A (2001) Estrogen prevents the lipopolysaccharide-induced inflammatory response in microglia. *J Neurosci* 21:1809–18
- Walder CE, Green SP, Darbonne WC, Mathias J, Rae J, Dinauer MC, Curnutte JT, Thomas GR (1997) Ischemic stroke injury is reduced in mice lacking a functional NADPH oxidase. *Stroke* 28:2252–8
- Wang Q, Santizo R, Baughman VL, Pelligrino DA (1999) Estrogen provides neuroprotection in transient fore-brain ischemia through perfusion-independent mechanisms in rats. *Stroke* 30:630–7
- Wen Y, Yang S, Liu R, Perez E, Yi KD, Koulen P, Simpkins JW (2004) Estrogen attenuates nuclear factor-kappa B activation induced by transient cerebral ischemia. *Brain Res* 1008:147–54
- Wise PM, Dubal DB, Wilson ME, Rau SW, Bottner M (2001) Minireview: neuroprotective effects of estrogen—new insights into mechanisms of action. *Endocrinology* 142:969–73
- Yang SH, Shi J, Day AL, Simpkins JW (2000) Estradiol exerts neuroprotective effects when administered after ischemic insult. *Stroke* 31:745–9