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Inducible nitric oxide synthase and estradiol exhibit complementary neuroprotective roles after ischemic brain injury

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Abstract

Estradiol-17 β exerts profound neuroprotective actions following cerebral ischemia through multiple molecular mechanisms. To examine the putative anti-inflammatory mechanisms employed by estradiol during stroke, we explored the interactions between estradiol and inducible nitric oxide synthase (iNOS) in both wildtype and iNOS knockout (iNOSKO) female mice following permanent middle cerebral artery occlusion (MCAO). Female mice were ovariectomized and treated with estradiol. One week later, they were subjected to MCAO, and then killed 24 hours later. Analysis of total, cortical and striatal infarct volumes confirmed that estradiol is neuroprotective in wildtype mice. Infarct volumes were also significantly smaller in female iNOSKO female mice, but estradiol did not further decrease injury. We found that one mechanism by which estradiol may act is by decreasing nitric oxide synthase 2 gene expression in the cortex and in the striatum of wildtype mice. These results show that the pro-inflammatory actions of iNOS exacerbate stroke-induced injury within the cortex and striatum, and that iNOS deletion is neuroprotective in ovariectomized and estrogen-replaced female mice.

Keywords

estradiol; estrogen; iNOS; stroke; inflammation; nitric oxide synthase; ischemia; middle cerebral artery occlusion

Stroke is a complex neurological disorder characterized by loss of cerebral blood flow, significant cell death, both necrotic and apoptotic in nature, and an enhanced inflammatory response (Dirnagl, et al., 1999). Women appear to be protected against stroke compared to men since studies show that premenopausal women have a lower incidence of stroke compared to age-matched males, but this protection is lost after the menopause (American Heart Association, 2004, Bushnell, et al., 2006). The biological basis of this protection has been thought to result from the neuroprotective properties of 17 β -estradiol. However, the results of the Women's Health Initiative (WHI), which concluded that estrogen treatment afforded no

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benefit or could increase risk for stroke in certain age groups, have encouraged reexamination of the actions of this hormone and its mechanisms of action (Anderson, et al., 2004, Rossouw, et al., 2007, Turgeon, et al., 2006, Turgeon, et al., 2004).

Inflammation plays an important role in the evolution of cerebral ischemia (reviewed by (Danton and Dietrich, 2003)). Inducible nitric oxide synthase, iNOS, is a critical regulator of the inflammatory response during stroke. Nitric oxide (NO) production resulting from induced *NOS2* gene expression and subsequent iNOS protein enzyme activation is a primary contributor to the post-ischemic inflammatory response. In turn, this sequence of events results in increased pro-inflammatory cytokine production, enhancement of leukocyte infiltration into the brain, and upregulation of adhesion molecules that contribute to both necrotic and apoptotic cell death (del Zoppo, et al., 2000, Zheng and Yenari, 2004). Knockout mouse models for iNOS (iNOSKO) demonstrate that iNOS expression and activation exacerbate injury in male mice following permanent middle cerebral artery occlusion (MCAO) (Iadecola, et al., 1997, Iadecola, et al., 1995, Sugimoto and Iadecola, 2002), but whether this protection extends to females is less clear (Loihl, et al., 1999, Park, et al., 2006). An emerging body of evidence strongly suggests that estradiol regulates inflammatory pathways in various disease paradigms (reviewed in (Maggi, et al., 2004)), but the underlying molecular mechanisms are diverse. To further elucidate the complex interactions between estradiol and inflammation during stroke, we examined the neuroprotective and potential anti-inflammatory properties of estradiol during permanent MCAO in female WT and iNOSKO mice.

Female C57BL/6J (wildtype, WT) and homozygous iNOS knockout (iNOSKO) mice (18–20 g; 8 weeks old) were purchased from Jax West. iNOSKO mice are backcrossed at least 8 generations onto the C57BL/6J background and were maintained as a viable stock at Jax West (West Sacramento, CA). All mice were ovariectomized (OVX) to eliminate endogenous ovarian steroids and implanted subcutaneously with a Silastic® capsule containing 180 µg/ml 17β-estradiol (E2) or sesame oil (vehicle). Circulating serum levels in mice using this technique (approximately 25 pg/ml) mimic levels observed during the estrous cycle (Dubal, et al., 2001). Following, ovariectomy, the mice were divided into the following treatment groups: 1) WT OVX+Oil, 2) WT OVX+E2, 3) iNOSKO OVX+Oil, 4) iNOSKO OVX+E2.

Seven days following ovariectomy, all mice underwent middle cerebral artery occlusion (MCAO). Mice were anesthetized with a mixture of chloral hydrate (350.0 mg/kg *i.p.*) and xylazine (4.0 mg/kg *i.p.*) and underwent permanent occlusion of the right middle cerebral artery via insertion of a 5/0 blue nylon suture as previously described (Dubal, et al., 2001). Body temperature was maintained at normothermia until recovery from anesthesia. Mice were killed 24 h after MCAO. Brains were removed and sectioned into 1-mm slices using a brain matrix, and these slices were further divided into two sets of alternating brain slices. One set was rapidly frozen onto glass slides using dry ice and stored at −80°C until needed for microdissection. The other set was stained for 15 minutes using 2% triphenyltetrazolium chloride (TTC), fixed in 10% phosphate buffered formalin, and quantified for infarct size. Brains were photographed, followed by quantification of total, cortical, and striatal injury using Metamorph 6.1 (Universal Imaging Corporation, Downingtown, PA). Individual infarct volumes, expressed as mm³, represent the sum of total and/or region-specific infarcts from all TTC-stained brain slices collected from a given mouse. The mean volume was then calculated from all mice within a given treatment group. For quantitative real-time PCR (qPCR), areas of cerebral cortex and striatum analyzed for gene expression were selected using the following criteria as previously described (Dubal, et al., 1999, Rau, et al., 2003). We first examined tissue from a 1-mm TTC-stained coronal section corresponding to the middle of the infarct. Then, corresponding 1mm³ sections of the cortex and striatum from the ipsilateral hemisphere was microdissected with a 1.0mm Harris Micropunch (Ted Pella, Redding, CA) for gene expression studies. Anatomically matched regions were then microdissected from the following treatment groups:

sham OVX+Oil, sham OVX+E2, WT OVX+Oil, WT OVX+E2, iNOSKO OVX+Oil, and iNOSKO OVX+E2. For all samples, anatomically similar regions were dissected to avoid collecting any infarcted tissue in the sample.

Next, total RNA was extracted from microdissected tissues using Ambion's RNAqueous®-Micro Kit (Ambion, Austin, TX) according to manufacturer's instructions. Approximately 50–100ng total RNA was reverse transcribed using the High Capacity cDNA Archive Kit with random primers (Applied Biosystems (ABI), Foster City, CA) followed by qPCR amplification. Each individual 50µl qPCR reaction contained: 2X Taqman Universal PCR master mix, with AmpErase UNG, 20X Taqman Assays-on-Demand Gene Expression primer/probe sets (all from ABI), and 20ng reverse-transcribed cDNA. PCR reactions were performed for the following primer sets of mouse genes: nitric oxide synthase 2 (*NOS2*) (Assay-on-Demand ID: Mm00440485_m1) and eukaryotic 18S, a standard housekeeping gene, (Assay-on-Demand ID: Hs99999901_s1) using an ABI Prism 7000 Sequence Detection System according to the manufacturer's instructions. Absolute quantification data was evaluated using ABI Prism 7000 SDS software, version 1.2.3. The relative expression (RQ) of all target genes was determined using the comparative $\Delta\Delta C_t$ method ((Livak and Schmittgen, 2001) and ABI PN 437109). Briefly, the C_t (threshold cycles) of *NOS2* were normalized to 18S, an endogenous control gene ($\Delta C_t = \Delta C_{t\ NOS2} - \Delta C_{t\ 18S}$) and then normalized to a calibrator sample (WT oil ipsilateral: 2A–B,E–F) or ipsilateral sham: 2C–D,G–H) such that $\Delta C_t = \Delta C_{t\ sample} - \Delta C_{t\ mean\ calibrator\ sample}$. Final results were expressed as a fold change ($2^{-\delta\delta C_t}$); data presented were subjected to a square root transformation followed by normalization of all values to the appropriate calibrator sample.

All data are expressed as mean $\pm$ SEM (8–14 mice were used for each treatment group (Oil or E2) per genotype (WT or iNOSKO) for all infarct measurements). The effects of estradiol and iNOS genotype treatment were analyzed using two-way ANOVA and post-hoc analysis was performed using Bonferroni's *post hoc* test with the Graphpad Prism 4.0 (Graphpad, San Diego, CA) and MDAS 2.0 (EsKay, Silver Spring, MD) statistical packages. Post hoc analyses were performed using two-tailed conditions unless otherwise indicated. All differences were considered significant at $p \leq 0.05$.

TTC stains live cells but is not taken up by dying cells and is a marker of ischemia-associated cell death; therefore alternating 1-mm brain slices were stained with TTC to determine the extent of infarcted tissue. Representative TTC-stained sections from wildtype (WT) oil-treated, WT-estradiol treated, iNOSKO oil-treated, and iNOSKO estradiol-treated mice (Figure 1A) show that infarct areas were larger in WT-oil treated mice than all other treatment groups, regardless of genotype. The TTC-stained infarcts in Figure 1A were quantified in sequential 1-mm brain sections containing visible infarcts. Quantification of total, cortical and striatal infarct volumes revealed the female iNOSKO mice are protected during stroke injury (Figure 1B). Total infarct volume was higher in WT oil-treated mice than in WT E2-treated mice, but total infarct volume was not significantly different between iNOSKO oil- and estradiol-treated mice. Furthermore, iNOSKO oil-treated mice had significantly smaller infarcts than WT-oil treated mice, providing clear evidence that iNOSKO mice are protected during stroke even in the absence of estradiol. In contrast, total infarct volume was not different between estradiol-treated WT or iNOSKO mice, suggesting that estradiol does not afford any additional neuroprotection in the female iNOSKO mouse.

Further analysis for quantification of the infarct in the cortex and striatum revealed that both estradiol and iNOS coordinately regulate infarct volume with great regional specificity in female mice. In the cortex and the striatum, the effects of treatment and genotype mirror what we observed in the whole brain, i.e. infarct volumes were larger in WT oil-treated mice than WT estradiol-treated mice. Infarct size was significantly smaller in iNOSKO mice, but there

was no significant difference between oil- and estradiol-treated iNOSKO mice. Likewise, striatal infarcts were also smaller in WT estradiol-treated mice compared to oil-treated mice, but iNOSKO mice displayed equivalent infarcts for both oil- and E2- treatment. Oil-treatment also produced smaller infarcts in iNOSKO mice compared to WT mice, but there were no striatal infarct differences observed with estradiol-treatment in both WT and iNOSKO mice.

Since we found that estradiol decreased infarct volume in WT mice, but did not provide any additional neuroprotection in iNOSKO mice, we tested whether one mechanism by which estradiol may influence iNOS was to suppress *NOS2* gene expression. Nitric oxide synthase 2 (*NOS2*) is the gene that encodes iNOS mRNA in mice, as well as other mammalian species (Kleinert, et al., 2003). In Figure 2 we used qPCR and the comparative Ct method to evaluate differences in *NOS2* gene expression between MCAO and sham ipsilateral cortex and striatum. In both the cortex and striatum, we found a significant decrease in *NOS2* mRNA following estradiol treatment in both MCAO- and sham-injured mice (cortex: Figure 2A–B, striatum: Figure 2E–F). Our results show that MCAO significantly increases *NOS2* gene expression in the cortex (Figure 2C–D), but the not in the striatum (Figure 2G–H). These findings suggest that estradiol-mediated suppression of *NOS2* gene expression inhibits the progression of ischemia-associated cell death mechanisms in the cortex, and to a lesser extent, the striatum, following permanent MCAO stroke injury.

In this study, we coupled a genetic approach using the iNOSKO mouse with physiological manipulation of estradiol levels to explore the potential interactions between estradiol and iNOS in protecting the brain against stroke-induced injury. Our study establishes three important points: 1) the presence of iNOS exacerbates stroke-induced brain injury in females; 2) estradiol protects against stroke-induced brain injury in WT ovariectomized mice, ovariectomized iNOSKO mice are protected compared to their WT counterparts, and that estradiol affords no further protection in the absence of iNOS gene expression; and 3) estradiol decreases *NOS2* gene expression after stroke-induced brain injury in females. These results demonstrate that iNOS plays an important role in the extent of cell death after stroke injury in females since knocking out this gene decreases infarct volume in the cortex and striatum of female iNOSKO mice.

The primary source of NO which contributes to post-ischemic secondary, apoptotic cell death is iNOS produced by glial cells, neurons, and infiltrating leukocytes (del Zoppo, et al., 2000, Zheng and Yenari, 2004). Knockout mouse models for *NOS2* (Iadecola, et al., 1997, Iadecola, et al., 1995, Nagayama, et al., 1999) as well as wildtype C57BL/6 mice treated with inhibitors of iNOS demonstrate that *NOS2* is a deleterious gene in models of both permanent and transient MCAO (Iadecola, et al., 1995, Sugimoto and Iadecola, 2002). Further studies have shown that the iNOS-associated net inflammatory response lasts significantly longer in permanent MCAO compared to transient MCAO models. Our laboratory and others have shown that physiological E2 treatment dramatically suppresses infarct volume and pro-inflammatory cytokine production during the post-ischemic inflammatory response that contributes to apoptotic cell death (Suzuki, et al., 2007, Suzuki, et al., 2006). An emerging body of recent studies suggests that physiological levels of E2 suppress the iNOS-mediated immune response in other neuroinflammatory paradigms. Nanomolar E2 concentrations decrease lipopolysaccharide (LPS) mediated nitric oxide (NO) production in human monocytes, a murine microglial cell line, and rat primary microglial cultures (Bruce-Keller, et al., 2000, Vegeto, et al., 2001, Vegeto, et al., 2004, Vegeto, et al., 2000).

Our results show that estradiol treatment downregulates *NOS2* gene expression in the cortex of both sham- and injured- mice, suggesting that one mechanism by which estradiol protects is by down-regulating a critical gene in the inflammatory response. We have previously reported that the striatum is dramatically protected by estradiol in female mice following

MCAO (Dubal, et al., 2006, Dubal, et al., 2001, Suzuki, et al., 2007), but the exact mechanism is unclear. The dramatic level of neuroprotection we observe in the striatum may be due to estradiol-mediated suppression of spreading depression from the cortex (Umegaki, et al., 2005, Witte, et al., 2000). In addition, a direct effect of estradiol in the striatum has been attributed to the activation of non-classical, membrane-bound estrogen receptors (Xiao, et al., 2003). Alternatively, the estradiol-mediated neuroprotection we observe in the striatum following MCAO may be attributed to changes in estrogen receptor alpha or beta expression that are induced after injury to suppress *NOS2* gene expression. Taken together, these findings strongly suggest that estrogens are important in suppressing brain inflammation even in the absence of injury and infer that postmenopausal women who do not use estrogen therapy may be in a chronic subclinical inflammatory state. This has been suggested in both preclinical and clinical studies (Franceschi, et al., 2000, Pfeilschifter, et al., 2002).

These results extend previous findings showing that iNOS expression exacerbates injury during stroke in male mice (Iadecola, et al., 1997). Our results do not agree with the previous findings of Loihl *et al.* (Loihl, et al., 1999) or Park *et al.* (Park, et al., 2006) who did not detect a neuroprotective effect of estradiol or iNOS in intact (Loihl, et al., 1999) or ovariectomized iNOSKO (Park, et al., 2006) mice when compared with their WT counterparts. Intact cycling mice, which are at various stages of the estrous cycle, are exposed to varying concentrations of both estradiol and progesterone, which may have differentially influenced the outcome of both studies. Additionally, Park *et al.* (Park, et al., 2006) used a transient MCAO model and assessed injury after 72h reperfusion. Thus, the discrepancies between the results of this study and ours may also be attributed to the injury model. Taken together, the seemingly contradictory results of these studies emphasize the critical importance of controlling the hormonal milieu and injury model when studying the neuroprotective actions of estradiol.

In summary, this study extends our knowledge of the role of iNOS in stroke by examining the complex and complementary interactions between estradiol and iNOS. Our findings lead to the conclusion that iNOS plays a dominant role in brain injury and that estradiol may protect through mechanisms that involve regulation of *NOS2* gene expression in the brain. Exploitation of the complementary relationship between estradiol and iNOS could lead to the development of potential stroke therapies for postmenopausal women.

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References

1. American Heart Association. Heart Disease and Stroke Statistics - 2005 Update. American Heart Association; Dallas: 2004. p. 1-63.
2. Anderson GL, Limacher M, Assaf AR, Bassford T, Beresford SA, Black H, Bonds D, Brunner R, Brzyski R, Caan B, Chlebowski R, Curb D, Gass M, Hays J, Heiss G, Hendrix S, Howard BV, Hsia J, Hubbel A, Jackson R, Johnson KC, Judd H, Kotchen JM, Kuller L, LaCroix AZ, Lane D, Langer RD, Lasser N, Lewis CE, Manson J, Margolis K, Ockene J, O'Sullivan MJ, Phillips L, Prentice RL, Ritenbaugh C, Robbins J, Rossouw JE, Sarto G, Stefanick ML, Van Horn L, Wactawski-Wende J, Wallace R, Wassertheil-Smoller S. Effects of Conjugated Equine Estrogen in Postmenopausal Women With Hysterectomy: The Women's Health Initiative Randomized Controlled Trial. *JAMA* 2004;291:1701-1712. [PubMed: 15082697]
3. Bruce-Keller AJ, Keeling JL, Keller JN, Huang FF, Camondola S, Mattson MP. Antiinflammatory effects of estrogen on microglial activation. *Endocrinology* 2000;141:3646-3656. [PubMed: 11014219]
4. Bushnell CD, Hurn P, Colton C, Miller VM, del Zoppo G, Elkind MSV, Stern B, Herrington D, Ford-Lynch G, Gorelick P, James A, Brown CM, Choi E, Bray P, Newby LK, Goldstein LB, Simpkins J.

Advancing the Study of Stroke in Women: Summary and Recommendations for Future Research From an NINDS-Sponsored Multidisciplinary Working Group. *Stroke* 2006;37:2387–2399. [PubMed: 16857945]

5. Danton GH, Dietrich WD. Inflammatory mechanisms after ischemia and stroke. *J Neuropathol Exp Neurol* 2003;62:127–136. [PubMed: 12578222]
6. del Zoppo G, Ginis I, Hallenbeck JM, Iadecola C, Wang X, Feurstein GZ. Inflammation and stroke: putative role for cytokines, adhesion molecules and iNOS in brain response to ischemia. *Brain Pathol* 2000;10:95–112. [PubMed: 10668900]
7. Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. *TINS* 1999;22:391–397. [PubMed: 10441299]
8. Dubal DB, Rau SW, Shughrue PJ, Zhu H, Yu J, Cashion AB, Suzuki S, Gerhold LM, Bottner MB, Dubal SB, Merchenthaler I, Kindy MS, Wise PM. Differential Modulation of Estrogen Receptors (ERs) in Ischemic Brain Injury: A Role for ER{alpha} in Estradiol-Mediated Protection against Delayed Cell Death. *Endocrinology* 2006;147:3076–3084. [PubMed: 16527848]
9. Dubal DB, Shughrue PJ, Wilson ME, Merchenthaler I, Wise PM. Estradiol modulates bcl-2 in cerebral ischemia: a potential role for estrogen receptors. *J Neurosci* 1999;19:6385–6393. [PubMed: 10414967]
10. Dubal DB, Zhu H, Yu J, Rau SW, Shughrue PJ, Merchenthaler I, Kindy MS, Wise PM. Estrogen receptor alpha, not beta is a critical link in estradiol-mediated protection against brain injury. *Proc Natl Acad Sci USA* 2001;98:1952–1957. [PubMed: 11172057]
11. Franceschi C, Bonafe M, Valensin S, Olivieri F, De Luca M, Ottaviani E, De Benedictis G. Inflamm-aging: An Evolutionary Perspective on Immunosenescence. *Ann NY Acad Sci* 2000;908:244–254. [PubMed: 10911963]
12. Iadecola C, Zhang F, Casey R, Nagayama M, Ross ME. Delayed reduction of ischemic brain injury and neurological deficits in mice lacking the inducible nitric oxide synthase gene. *J Neurosci* 1997;17:9157–9164. [PubMed: 9364062]
13. Iadecola C, Zhang F, Xu S, Casey R, Ross ME. Inducible nitric oxide synthase gene expression in brain following cerebral ischemia. *J Cereb Blood Flow Metab* 1995;15:378–384. [PubMed: 7536197]
14. Kleinert H, Schwarz PM, Forstermann U. Regulation of the Expression of Inducible Nitric Oxide Synthase. *Biol Chem* 2003;384:1343–1364. [PubMed: 14669979]
15. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 2001;28:402–408. [PubMed: 11846609]
16. Loihl AK, Asensio V, Campbell IL, Murphy S. 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* 1999;830:155–164. [PubMed: 10350569]
17. Maggi A, Ciana P, Belcredito S, Vegeto E. Estrogens in the Nervous System: Mechanisms and Nonreproductive Functions. *Ann Rev Physiol* 2004;66:291–313. [PubMed: 14977405]
18. Nagayama M, Aber T, Nagayama T, Ross ME, Iadecola C. Age-dependent increase in ischemic brain injury in wild-type mice and in mice lacking the inducible nitric oxide synthase gene. *J Cereb Blood Flow Metab* 1999;19:661–666. [PubMed: 10366196]
19. Park EM, Cho S, Frys KA, Glickstein SB, Zhou P, Anrather J, Ross ME, Iadecola C. Inducible nitric oxide synthase contributes to gender differences in ischemic brain injury. *J Cereb Blood Flow Metab* 2006;26:392–401. [PubMed: 16049426]
20. Pfeilschifter J, Koditz R, Pfohl M, Schatz H. Changes in proinflammatory cytokine activity after menopause. *Endocr Rev* 2002;23:90–119. [PubMed: 11844745]
21. Rau S, Dubal D, Bottner M, Wise P. Estradiol differentially regulates c-Fos after focal cerebral ischemia. *J Neurosci* 2003;23:10487–10494. [PubMed: 14627632]
22. Rossouw JE, Prentice RL, Manson JE, Wu L, Barad D, Barnabei VM, Ko M, LaCroix AZ, Margolis KL, Stefanick ML. Postmenopausal Hormone Therapy and Risk of Cardiovascular Disease by Age and Years Since Menopause. *JAMA* 2007;297:1465–1477. [PubMed: 17405972]
23. Sugimoto K, Iadecola C. Effects of aminoguanidine on cerebral ischemia in mice: comparison between mice with and without inducible nitric oxide synthase gene. *Neurosci Lett* 2002;331:25–28. [PubMed: 12359315]

24. Suzuki S, Brown CM, Dela Cruz CD, Yang E, Bridwell DA, Wise PM. Timing of estrogen therapy after ovariectomy dictates the efficacy of its neuroprotective and antiinflammatory actions. *Proc Natl Acad Sci USA* 2007;0610394104.
25. Suzuki S, Brown CM, Wise PM. Mechanisms of neuroprotection by estrogen. *Endocrine* 2006;29:209–215. [PubMed: 16785597]
26. Turgeon JL, Carr MC, Maki PM, Mendelsohn ME, Wise PM. Complex Actions of Sex Steroids in Adipose Tissue, the Cardiovascular System, and Brain: Insights from Basic Science and Clinical Studies. *Endocr Rev* 2006;27:575–605. [PubMed: 16763155]
27. Turgeon JL, McDonnell DP, Martin KA, Wise PM. Hormone Therapy: Physiological Complexity Belies Therapeutic Simplicity. *Science* 2004;304:1269–1273. [PubMed: 15166356]
28. Umegaki M, Sanada Y, Waerzeggers Y, Rosner G, Yoshimine T, Heiss WD, Graf R. Peri-infarct depolarizations reveal penumbra-like conditions in the striatum. *J Neurosci* 2005;25:1387–1394. [PubMed: 15703392]
29. Vegeto E, Bonincontro C, Pollio G, Sala A, Viappiani S, Nardi F, Brusadelli A, Vivani B, Ciana P, Maggi A. Estrogen prevents the lipopolysaccharide-induced inflammatory response in microglia. *J Neurosci* 2001;21:1809–1818. [PubMed: 11245665]
30. Vegeto E, Ghisletti S, Meda C, Etteri S, Belcredito S, Maggi A. Regulation of the lipopolysaccharide signal transduction pathway by 17[beta]-estradiol in macrophage cells. *J Steroid Biochem Mol Biol* 2004;91:59–66. [PubMed: 15261308]
31. Vegeto E, Pollio G, Ciana P, Maggi A. Estrogen blocks inducible nitric oxide synthase accumulation in LPS-activated microglia cells. *Exp Gerontol* 2000;35:1309–1316.
32. Witte OW, Bidmon HJ, Schiene K, Redecker C, Hagemann G. Functional differentiation of multiple perilesional zones after focal cerebral ischemia. *J Cereb Blood Flow Metab* 2000;20:1149–1165. [PubMed: 10950376]
33. Xiao L, Jackson LR, Becker JB. The effect of estradiol in the striatum is blocked by ICI 182,780 but not tamoxifen: pharmacological and behavioral evidence. *Neuroendocrinology* 2003;77:239–245. [PubMed: 12766324]
34. Zheng Z, Yenari MA. Post-ischemic inflammation: molecular mechanisms and therapeutic implications. *Neurol Res* 2004;26:884–892. [PubMed: 15727272]

Figure 1. Female iNOSKO mice are as equally protected following stroke injury as estradiol-treated WT mice

Total (A), cortical (B), and striatal (C) infarct volumes were quantified in WT and iNOSKO mice. (A) Estradiol reduces total infarct volume in WT mice (*, $p < 0.05$), but does not further suppress infarct size in iNOSKO mice. iNOSKO oil-treated mice were also protected during stroke compared to WT oil-treated mice (#, $p < 0.05$). (B) Cortical infarcts were also suppressed by estradiol in WT mice (*, $p < 0.05$), but estradiol did not exert any further infarct suppression in the iNOSKO cortex. Oil-treatment suppressed infarcts in iNOSKO compared to WT oil-treated mice (#, $p \leq 0.05$, one-tailed). (C) E2 treatment also suppressed striatal infarct volume in WT mice (*, $p < 0.05$), but striatal infarcts were not different in iNOSKO mice. Striatal infarct volumes in WT oil-treated mice were also larger than those in iNOSKO oil-treated mice (#, $p \leq 0.05$, one-tailed.) Data were analyzed using two-way ANOVA with Bonferroni's post hoc test: * indicates significant comparisons between oil and estradiol-treated mice within a genotype; # indicates significant comparisons within a treatment between genotypes; $n = 8-14$ mice/treatment/genotype. All values represent mean $\pm$ SEM.

Figure 2. *NOS2* is downregulated by estradiol

NOS2 gene expression was measured in cortical and striatal tissue from sham and WT-MCAO mice using qPCR. *NOS2* gene expression was significantly higher in the cortex (A-D) and striatum (E-H) from the ipsilateral side of the brain from in WT-MCAO (B-D, F-H) and WT-sham (A,E) oil-treated mice (cortex: effect of hormone, $p=0.023$; striatum: effect of hormone, $p=0.027$, two-way ANOVA). All data were analyzed using the comparative Ct method and expressed as a fold change in relative expression ($2^{-\Delta\Delta C_t}$); in panels A-B, E-F the oil-treated condition served as the calibrator, while in data panels C-D, G-H the sham treatment served as the calibrator. Final results were analyzed using two-way ANOVA with Bonferroni's post hoc test: * indicates significant comparisons between oil and estradiol-treated mice; $n=4-5$ mouse cortex or striatum tissue samples/treatment; all values expressed as mean $\pm$ SEM.