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Inducible Nitric Oxide Synthase in Neutrophils and Endothelium Contributes to Ischemic Brain Injury in Mice

Lidia Garcia-Bonilla,* Jamie M. Moore,* Gianfranco Racchumi,* Ping Zhou,* Jason M. Butler,† Costantino Iadecola,* and Josef Anrather*

NO produced by inducible NO synthase (iNOS) contributes to ischemic brain injury, but the cell types expressing iNOS and mediating tissue damage have not been elucidated. To examine the relative contribution of iNOS in resident brain cells and peripheral leukocytes infiltrating the ischemic brain, we used bone marrow (BM) chimeric mice in which the middle cerebral artery was occluded and infarct volume was determined 3 d later. iNOS<sup>−/−</sup> mice engrafted with iNOS<sup>+/+</sup> BM exhibited larger infarcts (44 ± 2 mm<sup>3</sup>; n = 13; mean ± SE) compared with autologous transplanted iNOS<sup>−/−</sup> mice (24 ± 3 mm<sup>3</sup>; n = 10; p < 0.01), implicating blood-borne leukocytes in the damage. Furthermore, iNOS<sup>−/−</sup> mice transplanted with iNOS<sup>−/−</sup> BM had large infarcts (39 ± 6 mm<sup>3</sup>; n = 13), similar to those of autologous transplanted iNOS<sup>+/+</sup> mice (39 ± 4 mm<sup>3</sup>; n = 14), indicating the resident brain cells also play a role. Flow cytometry and cell sorting revealed that iNOS is highly expressed in neutrophils and endothelium but not microglia. Surprisingly, postischemic iNOS expression was enhanced in the endothelium of iNOS<sup>+/+</sup> mice transplanted with iNOS<sup>−/−</sup> BM and in leukocytes of iNOS<sup>−/−</sup> mice with iNOS<sup>+/+</sup> BM, suggesting that endothelial iNOS suppresses iNOS expression in leukocytes and vice versa. To provide independent evidence that neutrophils mediate brain injury, neutrophils were isolated and transferred to mice 24 h after stroke. Consistent with the result in chimeric mice, transfer of iNOS<sup>+/+</sup>, but not iNOS<sup>−/−</sup>, neutrophils into iNOS<sup>−/−</sup> mice increased infarct volume. The findings establish that iNOS in both neutrophils and endothelium mediates tissue damage and identify these cell types as putative therapeutic targets for stroke injury.

**Materials and Methods**

Mice

All procedures were approved by the institutional animal care and use committee of Weill Cornell Medical College. Experiments were performed...
in 7- to 8-wk-old male iNOS−/− mice on a C57BL/6J background. DNAse I at 37°C for 45 min in an orbital shaker at 160 rpm. Cells were washed and subjected to discontinuous 70/25% Percoll (GE Healthcare, Piscataway, NJ) density gradient centrifugation (25 min, 800 × g, 4°C). Enriched mononuclear cells were collected from the interface, washed with HEPES–HBSS buffer, and resuspended in 2% FBS in PBS for staining and FACS analysis.

**FACS analysis and cell sorting**

Rat mAbs used for FACS analysis include: CD45-allophycocyanin (clone 30-F11), CD11b-PE (clone M1/70), Gr-1-PerCP-Cy5.5 (clone RB6-865), Ly6C-FITC (clone HK.1.4), CD45-FITC (ICAM-1, clone AF647) and CD11b-PE/VCAM-1 (clone 429/VMCA-1) from BioLegend (San Diego, CA). Isolated brain cells were stained on ice with predetermined optimal concentrations of Abs for 20 min and analyzed on an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). Dead cells and debris were gated out by forward light scatter and side scatter parameters. Cell populations were separated based on CD45 and Ly6C expression and further sorted by CD11b and Gr1 expression. Infiltrating leukocytes were identified as CD49b and further gated by CD11b and Gr1 markers. Neutrophils were identified as CD45+CD11b+Gr1b+iNOS−; CD45−CD11b−Gr1−iNOS+ sorting identifies monocytes/macrophages. Nonmyeloid leukocytes were considered as CD45+iNOS+/Gr1b; CD106−/−. Endothelial cells were identified as CD45−Ly6G−/− (23) and expression of CD54 (ICAM-1) and CD106 (VCAM-1) adhesion molecules on EC surface was also measured. Appropriate isotype control Abs. Fluorescence minus one staining and staining of negative populations were used to establish sorting parameters. Absolute cell numbers and frequencies were recorded. Additionally, in other experiments, the cell fractions of neutrophils, monocytes/macrophages, microglia and endothelial cells, identified using the same gating strategy as detailed above, were sorted on a FACSVantage cytometer (BD Biosciences) for mRNA analysis.

**Quantitative real-time PCR**

Cell fractions were collected in TRIzol LS (Invitrogen Life Technologies) and RNA was extracted according to the manufacturer’s instructions. RNA samples were treated with Rnase free Dnase (Roche, Indianapolis, IN) to remove DNA contamination. cDNA was produced from mRNA samples by using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative determination of gene expression for endothelial-leukocyte adhesion molecule 1 (Elam-1), Ly6G, and iNOS genes were performed on a Chroma 4 Detector (Bio-Rad, Hercules, CA) using a two-step cycling protocol. Hypoxanthine-guanine phosphoribosyltransferase (Hprt) was used to normalize gene expression. Primer sequences, purchased from Invitrogen Life Technologies, were as follows: Hprt, 5′-AGTTGTTG-GATACAGGCAAGAC-3′ and 5′-CTGCTGATAATTCACTTTAGAATG-3′; Elam-1, 5′-CTCTACTCTTGACTGCTG-TCT-3′; Ly6G, 5′-CCTGAGACTTCTCCTGACACA-3′; iNOS, 5′-TCACCAAG-GAGGAC/CATCAGGTAT-3′ and 5′-AGCTTCCAGGAGGAAGGTGAC-3′. qRT-PCR was conducted with cDNA in duplicate 15-µl reactions using the Maxima SYBR Green/ROX qPCR Master Mix (2×) (Thermo Scientific). The reactions were incubated at 50°C for 2 min and then at 95°C for 10 min. A PCR cycling protocol consisting of 15 s at 95°C and 1 min at 60°C for 45 cycles was used for quantification. Elam-1 and Ly6G relative expression levels were calculated by 2−ΔΔCT method (22). The number of both Hprt and iNOS gene copies in cell fractions was calculated from their respective cycle threshold, using an equation derived from a Hprt–cDNA or iNOS–cDNA plasmid standard curve, respectively, amplified in parallel. Data were expressed as iNOS mRNA copies relative to Hprt mRNA copies for each cell fraction.

**Western blot**

C57BL6 wild-type mice (n = 12) were subjected to transient MCAo and 3 d later, their brains were processed for flowmetric cell sorting of neutrophils, monocytes/macrophages, microglia, and endothelial cells, as described above. The cell fractions were pooled (n = 12 mice/cell fraction) and resolved on a 7% SDS-PAGE gel. Separated proteins were transferred onto a polyvinylidene difluoride membrane, and iNOS was immunodetected using a polyclonal-rabbit anti-iNOS Ab (1:100; number 153232; Abcam, Cambridge, MA). Immunoreactive bands were visualized using SuperSignal West Femto reagent (Thermo Scientific) on a chemiluminescent imager (LiCOR 420i gel blot scanner; LiCOR, Lincoln, NE). Lysates from Rv264.7 cells stimulated with LPS (10 ng/ml) and IFN-γ (100 U/ml) were used as a positive control of iNOS protein expression.
Adoptive cell transfer

Neutrophils were purified from BM of iNOS+/+ or iNOS−/− mice by immunomagnetic negative enrichment. Whole BM was flushed from femurs and tibias using a 26-gauge needle attached to a 10 ml PBS-filled syringe, and cells were dispersed by slow aspiration through a 20-gauge needle. Cell centrifugation (300 × g, 10 min, room temperature), the supernatant was removed by aspiration, and erythrolysis was performed using 5 ml ELB for 1 min, room temperature). Subsequently, 15 ml PBS was added, and the suspension was passed through a 40-μm cell strainer (catalog number 352340; BD Falcon). The strainer was then washed with 10 ml PBS. BM cells were centrifuged (300 × g, 10 min, room temperature) and resuspended in MACS buffer (PBS supplemented with 2% FBS and 2 mM EDTA; 100 μl/107 cells). An appropriate volume of biotinylated-Ab mixture (see Table I) and cells were purified with anti-biotin-microbeads, according to the manufacturer’s suggestions (Miltenyi Biotec). Diluted and centrifuged cell preparations contained >90% neutrophils as determined by flow cytometry (anti-Ly6G-PerCP-Cy5.5, 1A8 clone; BioLegend). Afterward, cells were washed, resuspended in sterile physiological saline (5 × 107 cells/100 μl), and administered by injection into the right retro-orbital sinus in anesthetized mice 24 h after MCAo (100 μl/mouse). Control animals received 100 μl saline.

Data analysis

Data are expressed as mean ± SE. Statistical differences in mortality were assayed by the Fisher’s exact test and χ² test. Intergroup differences in infarct volume, flow analysis, and mRNA expression were analyzed using a Student unpaired t test or one-way ANOVA with Dunnett’s posthoc analysis, as appropriate. Differences were considered statistically significant for p < 0.05.

Results

Mice groups and mortality

A total of 229 mice were used in this study. Of these, 52 naive mice were used for determination of infarct volume or FACS analysis of brain cells after MCAo. For adoptive transfer experiments, 25 mice were used as neutrophil donors and 40 mice were used as recipients, which were subjected to MCAo for infarct volume measurement. Six iNOS−/− mice were excluded due to inadequate ischemia–reperfusion. Three mice (one iNOS+/+ mouse and two iNOS−/− mice) died before 72 h and no differences were found between groups (Fisher’s test, p = 1). To generate chimeric animals, 10 mice were used as BM donors to obtain 102 chimeric mice, which were subjected to MCAo and used for infarct volume determination or FACS sorting. Four chimeric mice were excluded from the study because of inadequate ischemia–reperfusion (one iNOS+/+BM+/+ mouse, two iNOS−/−BM+/+ mice, and one iNOS−/−BM−/− mouse). Four chimeric mice (two iNOS−/−BM+/+ mice and two iNOS+/+BM−/− mice) died before 72 h. No differences were found in mortality rate among groups (χ², p = 0.4).

The protective effect of iNOS deletion is not due to reduced postischemic leukocyte infiltration

First, we sought to confirm that iNOS deficiency results in less severe ischemic injury after MCAo, as previously reported (11). iNOS−/− mice had smaller infarcts than iNOS+/+ mice 72 h after MCAo (Fig. 1A), a finding not because of differences in intra-ischemic CBF, which was similar in both groups (Fig. 1B). Because iNOS may influence the migration of inflammatory cells (24), we next examined whether the protection observed in iNOS−/− mice was the result of reduced leukocyte infiltration. To this end, we performed FACS analysis of brain cells in iNOS+/+ and iNOS−/− mice 48 h after MCAo (Fig. 2), the earliest time point of leukocyte infiltration after ischemia/reperfusion (Fig. 2B). FACS analysis showed no differences in the total number of infiltrating leukocytes (CD45+ cells) between iNOS+/+ and iNOS−/− mice, or in monocytes/macrophages and neutrophils (Fig. 2C). Similarly, no differences in the number of microglia and endothelial cells were observed between iNOS+/+ and iNOS−/− mice.

Furthermore, ICAM-1 and VCAM-1 expression in brain endothelial cells, which mediates postischemic neutrophil and monocyte/lymphocyte endothelial adhesion (25, 26), was similar in both groups 48 h after MCAo (Fig. 2C). Therefore, the protection afforded by iNOS deficiency is not secondary to reduced leukocyte infiltration after stroke.

iNOS expression in blood-borne leukocytes and resident brain cells contributes to brain injury after MCAo

To investigate the relative contribution to ischemic injury of iNOS in resident brain cells or BM-derived cells, we studied iNOS chimeric mice. Successful induction of chimism in BM-transplanted mice was verified by genomic analysis for the presence of the intact iNOS (NOS2) gene in peripheral blood leukocytes (Fig. 3A). Chimeric mice were subjected to MCAo and infarct volume was determined 72 h later (Fig. 3B). The infarct volume in iNOS−/− mice transplanted with iNOS−/−BM (iNOS−/−BM−/−) was smaller than that of iNOS+/+ mice transplanted with iNOS+/+BM (iNOS+/+BM+/+) by >90%, indicating that radiation and BM transplant did not influence the neuroprotection associated with the iNOS−/− genotype. However, in iNOS−/−BM+/+ chimeric mice, in which iNOS is present in peripheral leukocytes but not in resident brain cells, infarcts were larger than in iNOS+/−BM−/− (p < 0.01) and not different from those of iNOS−/−BM−/− mice (p > 0.05) (Fig. 3B). Surprisingly, in iNOS−/− mice transplanted with iNOS+/−BM (iNOS+/+BM−/−) the infarcts were not different from those of iNOS+/+BM+/+ mice (Fig. 3B), implicating also iNOS in resident brain cells in the damage. CBF changes during ischemia-reperfusion did not differ among groups (Fig. 3C), although a trend for increased hyperemia during early reperfusion was seen in iNOS+/+BM−/− mice. These data suggest that iNOS in BM-derived cells contributes to brain injury after MCAo, but that iNOS in resident brain cells may also play a role.

iNOS is expressed in infiltrating leukocytes and in endothelial cells, but not in microglia, after MCAo

The data presented above indicate that iNOS both in BM-derived and resident brain cells contribute to ischemic brain injury. We next sought to determine the cell types expressing iNOS after MCAo, which may be involved in the damage. Microglia and endothelial cells, resident brain cells with the greatest potential for iNOS expression (15, 27, 28), and infiltrating leukocytes (monocytes/macrophages and neutrophils) were isolated by FACS sorting from chimeric mice 72 h after MCAo, when postischemic iNOS expression is highest (29). Cell fractions were processed for qRT-PCR and iNOS mRNA expression was analyzed in each pop-
ulation (Fig. 4A). The purity of the sorted populations was evaluated by the expression of Elam-1 in EC and of Ly6G in neutrophils (Fig. 4B). In wild-type BM-transplanted mice, iNOS mRNA was induced in neutrophils, monocytes and endothelial cells, whereas iNOS expression was not observed in microglia. In iNOS−/− mice transplanted with iNOS+/+ BM, iNOS expression was enhanced in neutrophils and monocytes compared with iNOS+/+BM+/+ mice, but it was not detected in EC or microglia (Fig. 4A). Conversely, iNOS+/+ mice transplanted with iNOS−/− BM exhibited a significantly higher iNOS upregulation in EC compared with controls, whereas iNOS expression was not detected in microglia or infiltrating leukocytes (Fig. 4A). In addition, cell fractions isolated from wild type mice 72 h after MCAo were analyzed for iNOS expression at the protein level by western blotting (Fig. 4C). In agreement with mRNA data, iNOS protein expression was observed in monocytes/macrophages, neutrophils, and endothelial cells but not in microglia.

Adoptive transfer of iNOS+/+ neutrophils worsens ischemic injury in iNOS−/− mice

The data presented above indicate that iNOS is highly expressed in neutrophils infiltrating the ischemic brain. To determine whether iNOS in neutrophils contributes to tissue damage, we adoptively transferred iNOS+/+ or iNOS−/− neutrophils (5 × 10⁵ cells, i.v.; Table I) into recipient mice 24 h after MCAo (Fig. 5). iNOS+/+ mice receiving iNOS−/− neutrophils had smaller infarcts than iNOS+/+ mice receiving iNOS+/+ neutrophils. However, infarct volumes were larger in iNOS−/− mice receiving iNOS+/+ neutrophils and not statistically different from those of iNOS−/− mice receiving iNOS+/+ neutrophils (p > 0.05).

Discussion

This study provides several new findings. First, we demonstrated that the protection from ischemic injury observed in iNOS−/− mice is not due to reduced infiltration of blood-borne inflammatory cells, assessed quantitatively by FACS analysis. Second, using chimeric mice, we found that both resident brain cells (endothelial cells) and infiltrating leukocytes expressing iNOS contribute to postischemic brain injury. Third, we demonstrated that adoptive transfer of iNOS+/+ neutrophils is sufficient to offset in large part the protection in iNOS−/− mice, highlighting the pathogenic role of neutrophil iNOS in ischemic brain injury. Fourth, we found that suppression of iNOS expression in leukocytes enhances iNOS expression in endothelial cells and vice versa, unveiling a previously unappreciated reciprocal interaction regulating postischemic iNOS expression. These observations collectively provide new insight into the cellular expression of iNOS after ischemic stroke and its role in the ensuing brain damage.

iNOS is upregulated at the transcriptional level by the activity of NF-κB and Stat1α transcription factors among others (30). Accordingly, iNOS levels are normally very low, but after ischemia, iNOS transcription is induced resulting in the generation of large amounts of NO (31). Reacting with superoxide (O₂⁻), a free radical generated in the brain after ischemia/reperfusion (32), NO gives rise to the powerful oxidant peroxynitrite resulting in significant nitrosative damage of biological molecules, which, in

In infiltrating leukocytes (CD45hi), monocytes, and neutrophils showed similar levels in either iNOS+/+ or iNOS−/− 48 h after MCAo. No changes were also detected in the number of microglia or EC cells. In addition, the percentage of EC expressing ICAM-1 and VCAM-1 was similar in both groups. Values are mean ± SE, n = 6/group. ***p < 0.001, *p < 0.5 versus iNOS+/+ sham group.
turn, contributes to ischemic injury (33). However, another aspect of iNOS-derived NO function is its ability to regulate leukocyte recruitment to inflammatory sites (34). Although it is well established that inhibition (35, 36) or genomic deletion (37) of iNOS enhances endothelial leukocyte adhesion, in hepatic ischemic/reperfusion injury, iNOS-derived NO facilitates leukocyte trafficking into the injured brain. However, we did not find differences in the number of infiltrating leukocytes or changes in the level of discrete leukocyte populations involved in postischemic inflammation, such as neutrophils and monocytes (38). Furthermore, iNOS deletion did not affect the expression of endothelial ICAM-1 and VCAM-1, adhesion molecules involved in the postischemic transmigration of leukocytes across the blood–brain barrier (25, 26). Accordingly, our findings indicate that the deleterious effect of iNOS is not secondary to enhancement of leukocyte infiltration into the ischemic brain but to a direct action iNOS-expressing cells on the ischemic tissue.

iNOS immunoreactivity has been observed in microvessels and neutrophils after experimental stroke (12, 15, 16) and postmortem in stroke patients (17), but the pathogenic significance of iNOS in these cells was not established. To our knowledge, the present study is the first to provide evidence in support of a cell-specific pathogenic effect of endothelial and neutrophil iNOS on the postischemic brain. We found that both brain infiltrating neutrophils and monocytes express iNOS and reconstitution of an iNOS+/− host with iNOS+/+ BM was sufficient to counteract the protection and restore the injury to levels observed in wild-type mice. These findings, collectively, demonstrate that BM-derived cells, specifically neutrophils, represent a major source of the iNOS responsible for brain damage. Polymorphonuclear cells have long been implicated in the brain damage induced by postischemic inflammation through a wide variety of mechanisms (39–42). Our data suggest that iNOS-derived NO, which promotes lipid peroxidation, protein nitration, DNA damage, and blood–brain barrier breakdown (43), is a major neurotoxic molecule produced by neutrophils (44, 45). To provide further evidence that expression of iNOS in infiltrating leukocytes exacerbates ischemic injury, we adoptively transferred neutrophils into iNOS−/− mice.

We chose to transfer neutrophils because they express higher levels of iNOS than monocytes. Doing so was sufficient to increase the infarct volume to similar levels observed in iNOS−/− mice, confirming that neutrophil iNOS is a key player in ischemic brain damage. Our data show that neither transfer of iNOS−/− neutrophils into iNOS−/− mice, nor transfer of iNOS−/− neutrophils into iNOS−/− mice affected the infarct volume when compared with control iNOS−/− and iNOS+/+ mice, respectively. These findings indicate that the increased brain damage in iNOS−/− mice following iNOS−/− neutrophil transfer is most likely mediated...
by direct effects of iNOS-derived NO, rather than by an increase in the number of circulating neutrophils after adoptive transfer or microvascular clogging (46). Moreover, the number of transferred neutrophils (5 × 10^6 cells) is well below the total number of circulating neutrophils in C57BL/6 mice (1 × 10^8 cells) and can thus be considered to be within physiological limits (47).

We did not observe iNOS expression in microglia. iNOS expression can be induced in cultured microglia in the presence of LPS or inflammatory cytokines (48, 49) and in vivo after cerebral ischemia (50). However, iNOS expression in vivo has usually been attributed to microglia on the basis of immunohistochemical markers that cannot differentiate microglia from infiltrating macrophages (51). In contrast, FACS analysis, as used in this study, allows us to more clearly differentiate microglia from monocytes/macrophages based on quantitative assessment of their CD45 expression level (52). Using this approach, we were able to document iNOS expression in monocytes/macrophages but not in microglia. However, this observation does not exclude that possibility that microglial cells play a role in ischemic injury by producing other mediators (1, 53, 54). iNOS immunoreactivity has also been reported in astrocytes after ischemia in the postischemic period, but the role of astrocytic iNOS in the acute phase of the damage remains unclear because the expression occurs in the late postischemic period (3 d after ischemia) (16, 55).

Unexpectedly, we observed that iNOS−/+ mice transplanted with iNOS−−/− BM developed large infarcts despite of iNOS deficiency in infiltrating leukocytes. This effect was correlated with increased cerebral endothelial iNOS expression in the host, thus compensating for the lack of NO generation by infiltrating leukocytes. Therefore, it is conceivable that NO-generating leukocytes may limit endothelial iNOS expression after brain ischemia. Although the reasons for the increased endothelial iNOS expression in mice lacking leukocyte iNOS remain unclear, there is evidence for a negative feedback loop by which iNOS-derived NO suppresses iNOS induction by inhibiting NF-κB activity (56–58), providing a potential mechanisms for this effect. This finding has therapeutic implications in that it suggests that suppression of iNOS expression or activity in blood-borne leukocytes is not sufficient to confer protection from ischemic injury and that cerebral endothelial cells have to be targeted at the same time.

In conclusion, we have demonstrated that the harmful effects of iNOS in the ischemic brain are not mediated by enhancing the accumulation of inflammatory cells in the injured brain. Rather, iNOS in neutrophils infiltrating the ischemic brain and endothelial cells is required for the expression of its destructive effects. We also found that iNOS deletion only in leukocytes or endothelial cells is not sufficient to confer protection because lack of iNOS in one cell type enhances iNOS expression in the other. These findings provide further insight into the cellular bases through which iNOS exerts its deleterious effects on the brain and suggest specific cellular targets for therapeutic approaches harnessing the protective potential of iNOS inhibition.

**Disclosures**

The authors have no financial conflicts of interest.

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