Effect of Inducible Nitric Oxide Synthase on Cerebral Blood Flow after Experimental Traumatic Brain Injury in Mice

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ABSTRACT

Inducible nitric oxide synthase (iNOS) has been suggested to play a complex role in the response to central nervous system insults such as traumatic brain injury (TBI) and cerebral ischemia. In the current study, we quantified maps of regional cerebral blood flow (CBF) using an arterial spin-labeling magnetic resonance imaging (MRI) technique, at 24 and 72 h after experimental TBI in iNOS knockout (KO) and wild-type (WT) mice. Our hypothesis was that iNOS would contribute to the level of CBF at 72 h after experimental TBI in mice. Comparing anatomical brain regions of interest (ROIs) at 24-h post controlled cortical impact (CCI), there were significant reductions in CBF in the hemisphere, cortex, and contusion-rich area of the cortex of injured animals versus naive, regardless of genotype. Regional assessment of CBF at 72 h after injury demonstrated that recovery of CBF was reduced in the ipsilateral hippocampus, thalamus, and amygdala/piriform cortex in iNOS KO versus WT mice by 26%, 15%, and 21%, respectively; this attenuated recovery was restricted to structures outside the contusion. These regions with reduced CBF in iNOS KO mice represented ROIs where CBF in the WT was either numerically or statistically greater than that seen in respective WT naive, suggesting a contribution of iNOS to delayed posttraumatic hyperemia. However, pixel analysis denoted that the contribution of iNOS to CBF at 72 h was not limited to hyperemia flows. In conclusion, iNOS plays a role in the recovery of CBF after CCI in mice. Questions remain if this effect represents a homeostatic component of CBF recovery, pathologic vasodilatation linked to inflammation, or NO-mediated facilitation of angiogenesis.

Key words: arterial spin labeling; head injury; iNOS; magnetic resonance imaging; perfusion

INTRODUCTION

INDUCIBLE NITRIC OXIDE SYNTHASE (iNOS) has been suggested to play a complex role in the response to central nervous system (CNS) insults such as traumatic brain injury (TBI) and cerebral ischemia. iNOS is induced after injury and is expressed predominantly in resident and

infiltrating inflammatory cells and the cerebral vasculature (Clark et al., 1996; Iadecola et al., 1996), although neuronal expression has been reported in some models (Petrov et al., 2000). After experimental TBI in rats, iNOS mRNA expression is detected by 2 h and peaks at 6 h, while in mice a somewhat more delayed expression between 24 and 72 h after TBI is observed (Sinz et al.,

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1999). iNOS has been suggested to produce large quantities of nitric oxide (NO) since—unlike constitutive NOS isoforms—it is not regulated by calcium. Consistent with that notion, iNOS has shown by electron paramagnetic resonance (EPR) spectroscopy to contribute to ~50% of the NO detected in brain tissue at 72 h after experimental TBI comparing iNOS knockout (KO) versus wild-type (WT) mice (Bayir et al., 2005).

Overall, most studies suggest that, early after CNS injury, effects of iNOS may be detrimental since treatment with iNOS inhibitors or anti-sense reduces lesion volume, neuronal death, and a number of other markers of acute damage in experimental TBI (Wada et al., 1998a,b), spinal cord injury (Pearse et al., 2003), cryogenic brain injury (Stoffel et al., 2000), and stroke in both rats and mice (Iadecola et al., 1995; Iadecola et al., 1997). However, a dichotomous role for iNOS has been suggested in both experimental TBI and focal cerebral ischemia (Sinz et al., 1999; Bayir et al., 2005; Zhu et al., 2003), and iNOS has been recently shown to mediate ischemic tolerance in mice (Kawano et al., 2007). Several reports have suggested potential beneficial effects of iNOS, particularly at delayed time points after injury. For example, at 17–21 days after TBI, iNOS KO mice exhibit impaired recovery of cognitive outcome on the Morris water maze compared to WT (Sinz et al., 1999). In addition, iNOS KO mice were shown to have greater loss of brain levels of the antioxidant ascorbate versus WT type at 72 h after experimental TBI (Bayir et al., 2005). Similarly, in experimental stroke, iNOS KO mice exhibited complete absence of the robust neurogenesis response seen in the ipsilateral dentate gyrus at 48 h in WT (Zhu et al., 2003).

However, despite the known role of NO as a cerebral vasodilator, studies of the effect of iNOS on CBF after either experimental TBI or ischemia have been limited in contrast to those examining histological, functional, or biochemical endpoints (Willmot et al., 2005), and studies of the role of iNOS in delayed recovery of CBF are lacking. Iadecola et al (1997) reported no acute difference in CBF of the ischemic core or penumbra of iNOS KO versus WT mice assessed with laser-Doppler flowmetry during focal cerebral ischemia. To our knowledge, there is only one report on the role of iNOS in the CBF response to experimental TBI. Steiner et al. (2004), using the Marmarou impact acceleration model, reported that rats pre-treated with intracerebroventricular anti-sense iNOS oligodeoxynucleotides exhibited an exacerbation of the CBF reduction after TBI that was sustained in the initial 48 h after injury. CBF in that model was also assessed with laser-doppler flowmetry.

We recently reported the ability to assess regional CBF in mice—generating maps of CBF—using the continu-

ous arterial spin labeling (CASL) magnetic resonance imaging (MRI) method (Foley et al., 2005). In the current study, we took advantage of the utility of that approach to quantify maps of regional CBF at 24 and 72 h after experimental TBI in iNOS KO and WT mice. Our hypothesis was that iNOS would contribute to the level of CBF at 72 h after experimental TBI in mice.

METHODS

Animal Model

All experiments were approved by the Animal Care and Use Committees of Carnegie Mellon University and the University of Pittsburgh School of Medicine. iNOS KO (iNOS^{-/-}) and WT C57BL/6 (iNOS^{+/+}) mice were obtained from Jackson Laboratory (Bar Harbor, ME). The KO mice had been back-bred at least 10 generations. The animals were fed laboratory chow and water *ad libitum*. Mice were maintained under temperature controlled conditions with 12-h light/dark cycles. Knockout mice between the ages of 12 and 15 weeks were assigned into one of three groups: naive ($n = 8$), 24 h ($n = 8$), or 72 h ($n = 9$) after controlled cortical impact (CCI). Wild-type animals were divided into the same groups, naive ($n = 9$), 24 h ($n = 10$), and 72 h ($n = 9$) post CCI.

Controlled Cortical Impact

A mouse CCI model was used as previously described (Smith et al., 1995) with minor modifications (Whalen et al., 1999). Anesthesia was induced with 3% isoflurane and anesthesia was maintained with N₂O/O₂ (1:1) and 1–2% isoflurane via nose cone. Animals were placed in a stereotaxic holder and a temperature probe was inserted through a burr hole into the left frontal cortex and the left parietal bone was removed for trauma. Once brain temperature reached 37°C ± 0.5°C and was maintained at this temperature for 5 min, a vertically directed CCI was delivered (at a velocity of 4.0 m/sec and a depth of 1.0 mm). After injury, the bone flap was replaced, sealed with dental cement and the incision closed. Anesthesia was discontinued, and the animals were monitored in supplemental O₂ for 30 min and returned to their cages until MRI assessment.

Polymerase Chain Reaction Analysis

Genotypes of selected iNOS mice were determined by polymerase chain reaction (PCR) according to the protocol set by Jackson Laboratory, the animal provider. Briefly, 15 ng DNA was reacted with 10× PCR Buffer, 25 mM MgCl₂, 2.5 mM dNTPs, 5U/μl Taq Polymerase, H₂O and 20μM of the three primers designed by the man-

FIG. 1. Polymerase chain reaction (PCR) analysis of inducible nitric oxide synthase (iNOS) genotype. Wild-type mice show a single 108-basepair band, whilst iNOS knock-out mice exhibit a single 270-basepair band.

ufacturer. Cycling reaction times were denaturing at 94°C for 1.5 min, annealing at 94°C, 59°C, and 72°C, each 30 sec for 35 cycles and extension at 72°C for 2 min. PCR products were separated by electrophoresis on a 3% agarose gel. iNOS WT mice show a single 108-basepair band, while iNOS KO mice exhibit a single 270-basepair band (Fig. 1).

Magnetic Resonance Imaging Protocol

At the pre-selected time after CCI, anesthesia was induced as previously described (Foley et al., 2005), and mice were intubated and mechanically ventilated with 2% isoflurane and N₂O/O₂ (1:1). A femoral artery catheter was surgically inserted for continuous blood pressure monitoring and arterial blood sampling. A rectal temperature probe was inserted for monitoring and maintenance of temperature at $37.0 \pm 0.5^\circ\text{C}$ using a warm air heating system (SA Instruments, New York, NY). Mean arterial blood pressure (MABP) and heart rate were also monitored throughout image acquisition. Arterial blood gases were collected at the beginning and end of the studies; ventilator rate and tidal volume were adjusted to maintain arterial CO₂ tension (PaCO₂) of 30–45 mm Hg. Studies were discarded if the final PaCO₂ measurement was outside this range. Mice were placed onto a cradle in the prone position, and the head was secured with ear bars and an adjustable bite bar to limit motion.

Magnetic Resonance Image Acquisition

MR studies were performed on a 4.7-Tesla, 40-cm-bore Bruker AVANCE system, equipped with a 15-cm-diameter shielded gradient insert and a home-built saddle-type RF coil. Image acquisition parameters were

identical to those reported in a prior study (Foley et al., 2005). Briefly, *T*₂-weighted spin-echo images were used to verify the position of the center of the contusion, and were acquired with the following parameters: field of view 2.5 cm, 1-mm-slice thickness, 1.5-mm-interslice distance, *TR/TE* = 2500/40 msec, two averages, five slices, and a 128 × 70 matrix interpolated to 128 × 128. Perfusion studies were performed using a continuous arterial spin labeling (Detre et al., 1992; Williams et al., 1999), imaging technique (spin echo, 64 × 40 matrix interpolated to 64 × 64, *TR* = 2000 msec, summation of three echoes, *TE* = 10, 20, and 30 msec, and two averages). The labeling pulse for the inversion plane was positioned ± 2 cm from the perfusion detection plane. Spin-labeling efficiency (Zhang et al., 1993) was determined from intensities within the carotid arteries (gradient echo, 45° flip angle, eight averages, *TR/TE* = 100/9.6 msec, 256 × 256 matrix, and spin-labeling applied at ± 6 mm). The spin-lattice relaxation time of tissue water (*T*_{1obs}) (Hendrich et al., 1999) was measured from a series of spin-echo images (*TR* = 8000, 4300, 2300, 1200, 650, 350, 185, and 100 msec, *TE* = 9 msec, two averages, and a 64 × 40 matrix interpolated to 64 × 64).

Image Analysis

All image processing was performed with the Bruker ParaVision 3.0.2 image analysis software. Regions of interest (ROIs) defined the left (ipsilateral) and right (contralateral) hemisphere. Regions within each hemisphere including the cortex, hippocampus, thalamus and the amygdala/piriform cortex were also defined, guided by assignments from a mouse brain atlas (Sidman et al., 1971). Cortical ROIs were drawn to include the entire

TABLE 1. PHYSIOLOGICAL DATA^a

	WT naive (n = 9)	KO naive (n = 8)	WT 24-h post-CCI (n = 10)	KO 24 h post-CCI (n = 8)	WT 72-h post-CCI (n = 9)	KO 72-h post-CCI (n = 9)
MABP ^b	74.9 ± 15.4	91.9 ± 8.6 [#]	83.2 ± 8.0	80.8 ± 16.0	91.5 ± 9.9	91.2 ± 11.6
PaCO ₂	38.3 ± 7.2	35.9 ± 4.5	44.2 ± 14.2	36.9 ± 5.0	36.4 ± 3.0	36.0 ± 6.1
PaO ₂	229.7 ± 58.0	244.0 ± 44.0	212.8 ± 51.4	238.7 ± 22.7	249.3 ± 51.4	225.0 ± 50.5
pH	7.28 ± 0.03	7.36 ± 0.05*	7.25 ± 0.09	7.33 ± 0.05*	7.28 ± 0.06	7.36 ± 0.05*
Hematocrit	56.0 ± 10.0	49.0 ± 9.0	48.0 ± 7.0	44.0 ± 13.0	48.0 ± 9.0	43.0 ± 6.0
Heart rate ^b	435.0 ± 152.0	452.0 ± 71.0	404.0 ± 128.0	596.0 ± 86.0	431.0 ± 123.0	478.0 ± 102.0
Weight (g)	26.0 ± 2.0	27.0 ± 3.0	26.0 ± 2.0	28.0 ± 1.0*	26.0 ± 2.0	27.0 ± 3.0

^aData are mean ± SD.^bAt time of perfusion experiment.**p* < 0.05 versus naive.[#]*p* < 0.05 versus WT.

WT, wild-type; KO, iNOS knockout; CCI, controlled cortical impact; MABP, mean arterial blood pressure.

cortex within each hemisphere (cortex), or an area beginning 1 mm from midline to the midpoint of the arc of the cortex (cortex-CR). The cortex-CR region, after CCI, contains an area that has a large contribution from the contusion. Pixel by pixel maps of $(M_C - M_L) \cdot M_C^{-1}$ were generated from the perfusion data. M_C is the magnetization intensity from the control image and M_L is the magnetization intensity from the labeled image. The average of the two maps was computed and negative pixels in the brain were retained for quantification. $T_{1\text{obs}}$ maps were generated from the series of variable *TR* images by a three parameter non-linear fit to:

$$M(TR) = M_0 [1 - A \exp(-TR/T_{1\text{obs}})], \quad (1)$$

where $M(TR)$ is the signal intensity for each *TR* value, M_0 is the signal intensity at equilibrium, and A is the saturation correction factor. A mean value of the perfusion maps and $T_{1\text{obs}}$ were computed for each ROI. Regional CBF was then calculated from (Zhang et al., 1995):

$$\text{CBF} = \lambda \cdot (T_{1\text{obs}} \cdot 2\alpha)^{-1} \cdot (M_C - M_L) \cdot M_C^{-1} \quad (2)$$

where λ is the blood–brain partition coefficient of water, with a spatially constant value of 0.9 mL/g assumed (Herscovitch and Raichle, 1985), and α is the spin-labeling efficiency measured in the carotids. Pixel analysis was performed using ImageJ software (Abramoff et al., 2004). ROI's were segmented from each CBF map and histograms were generated for each timepoint and genotype.

Statistical Analysis

All data are expressed as mean ± standard deviation. Independent iNOS KO and WT mice were studied for each time point (naive, 24 h, and 72 h) and thus all phys-

iological data from KO and WT were compared at each time point using either a two-tailed Student's *t* test or analysis of variance (ANOVA). A probability of less than 0.05 (*p* < 0.05) was considered significant.

RESULTS

Physiology

Temperature at the time of CBF determination was maintained at 37.0°C ± 0.5°C for all mice. Arterial blood gases were measured at both the beginning and conclusion of each MRI experiment and averaged. Mean PaCO₂ and PO₂ values did not significantly differ between WT and KO mice or between injured and uninjured animals (Table 1). MABP at the time of CBF determination was 74.9 ± 15.4, 91.9 ± 8.6, 83.2 ± 8.0, 80.8 ± 16.0, 91.5 ± 9.9, 91.2 ± 11.6 mm Hg for the WT naive, KO naive, WT 24 h post-CCI, KO 24 h post-CCI, WT 72 h post-CCI, and KO 72 h post-CCI groups, respectively (Table 1). MABP for the KO naive mouse was significantly higher than that of the WT naive, but all MABP readings were in a physiologically acceptable range. Hematocrits did not differ between groups (Table 1). The average weight of the animals used in the KO 24 h post-CCI group was slightly, albeit significantly higher than WT. The mean pH of the iNOS KO groups was modestly, albeit significantly higher than that of the WT mice, but all were in a physiologically acceptable range.

$T_{1\text{obs}}$ Maps, Spin Labeling Efficiency (α), and Regional Cerebral Blood Flow

Mean $T_{1\text{obs}}$ values for all studies are listed in Table 1. After CCI, $T_{1\text{obs}}$ values ipsilateral to the injury were sig-

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TABLE 2. REGIONAL $T_{1\text{obs}}$ (SEC) AND ARTERIAL SPIN LABELING EFFICIENCY (α)^a

	WT naïve (n = 9)	KO naïve (n = 8)	WT 24-h post-CCI (n = 10)	KO 24 h post-CCI (n = 8)	WT 72-h post-CCI (n = 9)	KO 72-h post-CCI (n = 9)
$T_{1\text{obs}}$						
Hemisphere						
Left	1.62 ± 0.08	1.64 ± 0.07	2.01 ± 0.24*	2.09 ± 0.33*	2.11 ± 0.62*	1.78 ± 0.16*
Right	1.63 ± 0.10	1.67 ± 0.05	1.71 ± 0.12	1.69 ± 0.05	1.74 ± 0.16	1.63 ± 0.11
Cortex						
Left	1.66 ± 0.09	1.67 ± 0.07	2.54 ± 0.88*	2.68 ± 0.75*	2.66 ± 1.30*	2.10 ± 0.32*
Right	1.69 ± 0.12	1.69 ± 0.06	1.78 ± 0.18	1.76 ± 0.10	1.84 ± 0.31	1.70 ± 0.12
Cortex-CR						
Left	1.64 ± 0.09	1.66 ± 0.06	3.00 ± 1.59*	3.26 ± 1.86*	3.66 ± 2.67*	2.23 ± 0.47*
Right	1.66 ± 0.13	1.68 ± 0.07	1.72 ± 0.14	1.76 ± 0.15	1.88 ± 0.44	1.66 ± 0.15
Thalamus						
Left	1.44 ± 0.06	1.50 ± 0.06	1.56 ± 0.10*	1.46 ± 0.04 [#]	1.48 ± 0.12	1.41 ± 0.08 [#]
Right	1.45 ± 0.08	1.53 ± 0.08 [#]	1.54 ± 0.11	1.50 ± 0.06	1.51 ± 0.14	1.46 ± 0.11
Amygdala/piriform cortex						
Left	1.80 ± 0.16	1.78 ± 0.14	1.85 ± 0.19	1.73 ± 0.05	1.79 ± 0.21	1.69 ± 0.11
Right	1.80 ± 0.14	1.82 ± 0.14	1.78 ± 0.03	1.77 ± 0.06	1.80 ± 0.24	1.74 ± 0.17
Hippocampus						
Left	1.69 ± 0.15	1.69 ± 0.07	1.99 ± 0.23*	1.95 ± 0.29*	1.73 ± 0.16	1.76 ± 0.23
Right	1.74 ± 0.17	1.79 ± 0.16	1.80 ± 0.17	1.87 ± 0.16	1.78 ± 0.20	1.70 ± 0.19
α	0.68 ± 0.04	0.70 ± 0.05	0.69 ± 0.04	0.67 ± 0.03	0.67 ± 0.04	0.68 ± 0.05

^aData are mean ± SD.

* $p < 0.05$ versus naïve.

[#] $p < 0.05$ versus WT.

WT, wild-type; KO, iNOS knockout; CCI, controlled cortical impact.

nificantly elevated. The $T_{1\text{obs}}$ of the contusion rich area of the cortex was 3.10 ± 1.59 sec on the injured side versus 1.72 ± 0.13 sec on the contralateral side for WT mice 24 h post-CCI, the KO animals had similar values (Table 2). $T_{1\text{obs}}$ was 1.64 ± 0.09 and 1.66 ± 0.13 sec in the ipsilateral and contralateral contusion rich area of the cortex of the WT mice, again the KO mice were similar (Table 2). $T_{1\text{obs}}$ values were also significantly increased in the ipsilateral hemisphere, cortex and hippocampus of WT and KO mice 24 h post-CCI and the ipsilateral hemisphere, cortex and cortex-CR of WT and KO mice 72 h post-CCI (Table 2). The mean values for α were 0.68, 0.7, 0.69, 0.67, 0.67, and 0.68 for the naïve WT and KO, WT and KO 24 h post-CCI, and WT and KO 72 h post-CCI groups, respectively, and did not statistically differ between groups (Table 2).

Representative CBF maps obtained from naïve mice and after CCI are shown in Figure 2. Regions with low perfusion generally corresponded to areas with elevated $T_{1\text{obs}}$ values (Table 2). Mean regional CBF values for all studies are shown in Figure 3. Comparing anatomical brain ROIs at 24 h post-CCI, there were significant reductions in CBF in the injured animals versus naïve, re-

gardless of genotype. Values for the ipsilateral hemisphere of WT animals at 24 h post-CCI were reduced by 31%, while the cortex and cortex-CR flows were reduced by 57% and 76%, respectively, compared to naïves. KO mice at 24 h after CCI experienced similar decreases in CBF as the WT of 38%, 67%, and 85% in the hemisphere, cortex and cortex-CR, respectively; however, the KO mice also exhibited a significant reduction of CBF in the hippocampus versus respective naïve (Fig. 3). In contrast, regional assessment of CBF at 72 h after injury demonstrated that recovery of CBF was reduced in the ipsilateral hippocampus, thalamus, and amygdala/piriform cortex in iNOS KO versus WT mice by 26%, 15%, and 21% respectively. Of note, these three regions with reduced CBF in iNOS KO versus WT at 72 h represented ROIs where CBF in the WT was either numerically or statistically greater than that seen in respective WT naïve—suggesting posttraumatic hyperemia. The attenuated recovery of CBF in iNOS KO versus WT at 72 h after CCI was restricted to these three ROIs since CBF in iNOS KO and WT mice at 72 h post-CCI in the cortex and cortex-CR regions did not differ between genotypes and remained significantly lower than naïves (Fig. 3).

FIG. 2. Representative cerebral blood flow (CBF) maps of wild-type (WT) and inducible nitric oxide synthase (iNOS) knock-out (KO) mice without injury (naïve) and at 24 h and 72 h after controlled cortical impact (CCI) injury. There was no difference in CBF in any region of interest (ROI) in naïve WT versus iNOS KO mice. After injury, maps show spatial heterogeneity. At 24 h after injury, a region of reduced CBF is readily identified in the contused cortex of both WT and iNOS KO mice. Similarly, at 24 h after CCI, overall hemispheric CBF was reduced ipsilateral to injury in both WT and iNOS KO mice. At 72 h after injury, recovery of CBF was attenuated in iNOS KO versus WT. Maps represent the best example of the average CBF for each group. Pixels outside the brain have been assigned zero intensity.

To gain additional insight of the effect of iNOS in these different brain regions we examined the distribution of blood flow in selected brain regions in the WT versus the KO. Pixel analysis of CBF maps from all animals at 72 h show that for the contralateral side there is no obvious difference between genotype (Fig. 4A). The most interesting finding came when the ipsilateral hippocampus was analyzed (Fig. 4B). Pixels with a CBF below 100 mL/100 g/min were only seen in the iNOS KO mice. This is consistent with the notion that CBF recovery is blunted in the ipsilateral hippocampus, and that this effect is not limited to the attenuation of hyperemic flows.

DISCUSSION

Our data suggest that iNOS plays a role in the normal recovery of CBF after CCI in mice. iNOS expression may contribute as much as 26% to the observed CBF following injury and also depends on the time after injury and brain region. Attenuated recovery of CBF in iNOS KO versus WT occurred predominantly at 72 h after injury and was restricted to structures outside of the contusion.

Curiously, those regions generate supra-normal CBF values in WT at 72 h after injury.

After TBI in rats using a weight-drop model iNOS positive cells are seen around the injury site at 4–6 h, expression peaks at ~24 h, and gradually decreases (Gahm et al., 2000). In immature rats using a weight-drop model, iNOS expression was noted at low levels as soon as 2 h after injury but marked at 24 and 48 h (Clark et al., 1996). Wada et al. (1998a,b) saw increased iNOS activity at 3 days after fluid-percussion injury with the most dramatic increase occurring at 7 days in adult rats. The predominant cell types expressing iNOS are neutrophils (Clark et al., 1996; Gahm et al., 2000), macrophages (Gahm et al., 2000; Petrov et al., 2000) and vascular smooth muscle cells, which suggests that iNOS may play a role in cerebrovasodilation (Clarke et al., 1996). Petrov et al. (2000) found that 24 h after TBI, using a weight drop model in rats, there is also iNOS expression in injured neurons. It is interesting to note that in the naïve animals there was no difference in CBF between WT and KO. This is consistent with the notion that iNOS is induced and not present in significant quantities in the normal brain.

It is not clear if the effect of iNOS on CBF at 72 h af-

FIG. 3. Regional cerebral blood flow (CBF) values (mean $\pm$ SD) from studies with naive wild-type (WT; $n = 9$) and knock-out (KO; $n = 8$) mice (A), WT 24 h ($n = 10$), and KO 24 h ($n = 8$) after controlled cortical impact (CCI) (B), and WT 72 h ($n = 9$) and KO 72 h ($n = 9$) post-CCI (C). Standard deviation (SD) in CBF represents the intra-group variability computed from $(M_C - M_L) \cdot M_C^{-1}$. * $p < 0.05$ versus naive and # $p < 0.05$ versus WT by Student's *t*-test.

FIG. 4. Pixel analysis of cerebral blood flow (CBF) maps for all inducible nitric oxide synthase (iNOS) wild-type (WT) and knockout (KO) mice at 72 h after experimental traumatic brain injury (TBI). Contralateral (**A**) and ipsilateral (**B**) hippocampus.

ter injury in our model represents enhanced recovery or a pathological process. In this same mouse model, we previously reported that the iNOS KO exhibited poorer functional recovery than the WT between 17 and 21 days after injury, as assessed by Morris water maze performance (Sinz et al., 1999), but no significant difference between genotypes was noted in contusion volume at 21 days. Conversely, administration of the iNOS inhibitor aminoguanidine (AG) reduced the number of necrotic neurons seen after fluid percussion injury in rats, but did not reduce contusion volume (Wada et al., 1998a,b). The delayed use of the more selective iNOS inhibitor 1400W, given at 18 or 24 h after injury, improved histopathological outcome (Jafarian-Tehrani et al., 2005). Louin et al. (2006) investigated the effect of three different iNOS

inhibitors, each with increasing selectivity—AG, *L*-*N*-iminoethyl-lysine (*L*-NIL), and 1400W—after TBI and showed that each inhibitor improved neurological function at 24 h (Louin et al., 2006). However, sustained treatment with AG for 5 days or *L*-NIL for 1.5 days worsened histological outcome at 21 days after CCI in rats (Sinz et al., 1999). Taken together, iNOS appears to mediate detrimental effects early—in the initial 24 h—while mediating beneficial actions at more delayed time points. The observed deficit in CBF recovery at 72 h but not 24 h after TBI in the setting of iNOS deficiency in our study is consistent with a more dominant role for iNOS in the recovery phase, rather than the acute phase. Given that iNOS induction after CCI in mice is a fairly delayed event, and that many early events in the secondary injury

cascade, such as acute energy failure, play an important role in tissue loss, it is reasonable that increased contusion volume was not observed in the iNOS KO versus WT.

Using EPR spectroscopy, we previously reported (Bayir et al., 2005) that the NO levels are ~50% lower in the injured hemisphere at 72 h after CCI in iNOS KO versus WT mice in this exact model. Thus, loss of a direct effect of NO in iNOS KO mice likely explains our findings. There are several possibilities to consider. Induction of iNOS and production of iNOS-derived NO in the regions outside of the contusion may mediate (1) direct vasodilatory effects in the delayed recovery of CBF, or (2) pathological hyperemic CBF as part of the inflammatory process in brain, and/or angiogenesis in brain regions recovering and/or remodeling after CCI.

Rats pre-treated with anti-sense iNOS oligodeoxynucleotides exhibited an exacerbation of the CBF reduction after TBI, produced by impact acceleration, which was sustained in the initial 48 h (Steiner et al., 2004). An interaction between iNOS and endothelin-1 (ET-1) was suggested to mediate this CBF reduction in those studies since the posttraumatic increases in ET-1 levels were augmented between 4 and 48 h after injury. A synergistic loss of vasodilatory effects of NO and enhanced expression of ET-1 in iNOS KO mice was proposed to mediate this CBF effect, since NO exhibits an inhibitory influence on activator protein-1 (AP-1)—a known up-regulator of ET-1 expression. However, the work by Steiner et al (2004) had several limitations including the use of laser-Doppler flow assessment, and thus provided only a relative flow assessment. There was also no quantification of relative flow in that study or statistical comparison between groups, in lieu of presentation of the slope of the regression line of mice in each group over time—making it difficult to appreciate the magnitude of the effect of inhibition of iNOS on CBF. Nevertheless, a contribution of iNOS to ameliorating posttraumatic hypoperfusion was supported. We did not assess ET-1 levels, and thus cannot comment on possible synergism between loss of NO and increases in ET-1 in our model.

In our study, the effects of genotype on CBF were largely restricted to blunting of delayed posttraumatic increases in CBF normally observed in subcortical ROIs such as thalamus and amygdala. iNOS has been shown to mediate cerebral hyperemia in the inflammatory response, such as that observed in rat brain in response to endotoxin administration (Okamoto et al., 1998). There is robust accumulation of local and infiltrating inflammatory cells after injury in the mouse CCI model supporting this possibility (Carlos et al., 1997; Bayir et al., 2005). Given the severity of the effect of CCI on the contused cortex, it would also be reasonable to speculate that

the lack of effect of genotype on CBF within the contusion represents lack of responsivity of the severely damaged vasculature to NO in that region. Finally, our pixel analysis in hippocampus revealed that the effect of iNOS on CBF was not limited to a reduction in hyperemic flows—given that pixels with CBF values of <100 mL/100g/min were seen only in the iNOS KO. Indeed, pixels with CBF values of <50 mL/100g/min were seen. It is not clear, however, whether this finding is mediating, in part, the impaired water maze performance previously reported after CCI in this genotype (Sinz et al., 1999). Indeed, the implications of the effect of iNOS on CBF recovery after CCI remain to be defined.

Over-expression of iNOS was recently shown to induce angiogenesis and regulate the degree of perfusion in tumors (Cullis et al., 2006). Similarly, a critical role for iNOS in neurogenesis after focal cerebral ischemia is known (Zhu et al., 2003). These effects may be mediated by cGMP as suggested by Zhang et al. (2002). Given the rapid time course that has been reported for angiogenesis in experimental CNS injury, where a significant increase in the number of vessels can be seen in post-ischemic brain at 3 days (Hayashi et al., 2003), we cannot rule out the possibility that the attenuated CBF response seen in iNOS KO mice results from the preclusion of iNOS-mediated angiogenesis.

Whether iNOS-mediated effects on CBF after TBI are beneficial, detrimental, or an epiphenomenon remains unclear. Cerebral metabolic rate, as assessed by deoxyglucose utilization, at 24 or 72 h after CCI in rodents is generally reduced (Hovda et al., 1995), suggesting that hyperemic flows at 72 h after injury may be pathologic. However, a regional assessment of cerebral metabolic rate was not carried out in this study. Similarly, the specific cell types mediating the effects of iNOS on CBF after TBI remain to be determined. These represent logical areas for future investigation.

There are limitations to this work. Arterial pH was slightly lower in WT versus KO mice for each comparison—naïve, 24 h, and 72 h. We do not anticipate that this difference would importantly influence CBF since PaCO₂ was controlled and did not differ between groups. This suggests that the difference was related to a mild metabolic acidosis in WT that was blunted in the KO. The reason for this finding remains unclear; however, systemic effects of loss of iNOS-derived NO cannot be ruled out. MABP was also higher in the naïve KO, which was a surprising finding. However, all mice had a MABP in the physiologically acceptable range and no difference in MABP was seen between genotypes after TBI. Second, because of the need for invasive blood gases and blood pressure monitoring, it was not possible to serially study mice—despite the fact that the CASL method af-

fords the advantage of being a non-invasive approach. Assessing the temporal profile of CBF across time in individual mice would have been desirable. Finally, we did not measure NO levels in these mice to confirm an effect of iNOS on NO production in brain; however, we previously reported a ~50% reduction in NO levels by EPR spectroscopy in the injured hemisphere in this model in iNOS KO versus WT mice (Bayir et al., 2005).

Anesthetic preconditioning with isoflurane is recognized to be neuroprotective. Pretreatment with isoflurane for a period of 30 min to 3 h induces a time-dependent increase in iNOS protein expression. Inhibition of iNOS abolished the isoflurane preconditioning induced neuroprotection (Kapinya et al., 2002; Zhao and Zuo, 2004). Although we used isoflurane anesthesia in our studies, at 24 h post-CCI when iNOS expression from isoflurane induction is reported to be at a peak (Kapinya et al., 2002), there was no important difference in CBF between iNOS WT and KO mice. This suggests that it is unlikely that isoflurane played a key role in our findings. However, studies using additional anesthetics are warranted.

In conclusion, iNOS plays a role in the recovery of CBF after CCI in mice. Attenuated recovery of CBF in iNOS KO versus WT predominantly occurred at 72 h after injury and was restricted to structures outside of the contusion. Whether this effect of iNOS represents a homeostatic component of CBF recovery, pathologic vasodilatation linked to inflammation, or results from NO-mediated facilitation of angiogenesis remains to be determined.

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