Published in final edited form as:
Neuroscience. 2007 January 5; 144(1): 56–65.

Influence of Duration of Focal Cerebral Ischemia and Neuronal Nitric Oxide Synthase on Translocation of Apoptosis-Inducing Factor to the Nucleus

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Abstract

Translocation of apoptosis-inducing factor (AIF) from the mitochondria to the nucleus can play a major role in neuronal death elicited by oxidant stress. The time course of nuclear translocation of AIF after experimental stroke may vary with the severity of injury and may be accelerated by oxidant stress associated with reperfusion and nitric oxide (NO) production. Western immunoblots of AIF on nuclear fractions of ischemic hemisphere of male mice showed no significant increase with 1 hour of middle cerebral artery occlusion and no reperfusion, whereas increases were detectable after 6 and 24 hours of permanent ischemia. However, as little as 20 minutes of reperfusion after 1 hour of middle cerebral artery occlusion resulted in an increase in nuclear AIF coincident with an increase in poly(ADP-ribose) polymer (PAR) formation. Further nuclear AIF accumulation was seen at 6 and 24 hours of reperfusion. In contrast, 20 minutes of reperfusion after 2 hours of occlusion did not increase nuclear AIF. In this case, nuclear AIF became detectable at 6 and 24 hours of reperfusion. With brief occlusion of 30 minute duration, nuclear AIF remained undetectable at both 20 minutes and 6 hours and became evident only after 24 hours of reperfusion. Inhibition of neuronal NO synthase attenuated formation of PAR and nuclear AIF accumulation. Gene deletion of neuronal NO synthase also attenuated nuclear AIF accumulation. Therefore, reperfusion accelerates AIF translocation to the nucleus when focal ischemia is of moderate duration (1 hour), but is markedly delayed after brief ischemia (30 minutes). Nuclear translocation of AIF eventually occurs with prolonged focal ischemia with or without reperfusion. Neuronally-derived NO is a major factor contributing to nuclear AIF accumulation after stroke.

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Cellular Neuroscience Section Editor: Dr. Constantino Sotelo

Keywords

apoptosis; middle cerebral artery; mouse; poly(ADP-ribose); reperfusion; stroke

INTRODUCTION

Apoptosis inducing factor (AIF) is an oxidoreductase protein that normally is associated with mitochondria. However, translocation of AIF to the nucleus results in irreversible cell death signaling that is independent of caspase activity (Susin et al., 1999;Daugas et al., 2000). In neurons, AIF translocation to the nucleus appears to play an important role in NMDA and kainate excitotoxicity (Yu et al., 2002;Wang et al., 2004;Cheung et al., 2005) and in oxygen-glucose deprivation (Cao et al., 2003;Plesnila et al., 2004;Culmsee et al., 2005). In brain, nuclear AIF translocation has been reported after hypoglycemic coma (Ferrand-Drake et al., 2003), global cerebral ischemia (Cao et al., 2003), hypoxia-ischemia in postnatal rats (Zhu et al., 2003) and mice (Matsumori et al., 2005), and traumatic brain injury (Zhang et al., 2002).

Translocation of AIF has also been demonstrated with focal cerebral ischemia (Ferrer et al., 2003;Komjati et al., 2004). After permanent occlusion of the distal middle cerebral artery (MCA) in rats, significant increases in AIF in nuclear subcellular fractions occurred at 48 hours, although localized increases in cortical border regions were evident at 8 hours (Zhao et al., 2004). With 45 minutes of transient MCA occlusion (MCAO) in mice, significant increases in nuclear AIF in cortex occurred by 1 hour (Plesnila et al., 2004) and 2 hours (Culmsee et al., 2005). However, the time course of nuclear AIF translocation has not been evaluated systematically after varying durations of focal ischemia, in which delayed apoptosis is known to be evident after brief ischemia and necrotic cell death rapidly develops with prolonged ischemia (Kametsu et al., 2003). Nor has it been determined if reperfusion accelerates AIF translocation. The first objective of the present study was to investigate the time course of AIF translocation after varying durations of MCAO.

Translocation of AIF can be triggered by poly(ADP-ribose) polymerase (PARP) activity (Yu et al., 2002;Wang et al., 2004). Activation of PARP, in turn, is triggered by damage to DNA from oxidizing agents, such as peroxynitrite formed from nitric oxide (NO) and superoxide (Zhang et al., 1994). Gene deletion of neuronal NO synthase (nNOS) results in decreased formation of peroxynitrite (Eliasson et al., 1999) and decreased formation of poly(ADP-ribose) polymers (PAR) by PARP activity after MCAO (Endres et al., 1998). The second objective of the present study was to determine the role of nNOS in nuclear AIF translocation.

The primary aims of the present study were 1) to compare the time course of AIF translocation to the nucleus with graded injury after 30, 60, or 120 minutes of MCAO in mice, 2) to determine if reperfusion accelerates AIF translocation, compared to permanent MCAO, using the same technique for MCAO, and 3) to determine if inhibition of nNOS with 7-nitroindazole (7-NI) or gene deletion of nNOS ($nNOS^{-/-}$) reduces the accumulation of AIF in the nucleus after MCAO.

EXPERIMENTAL PROCEDURES

Focal cerebral ischemia model

All procedures on mice were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Johns Hopkins University Animal Care and Use Committee. For experiments on the time course of AIF translocation, C57Bl/6 mice were used. Approximately 300 male mice weighing between 20 and 28 g were

anesthetized initially with 2% halothane and maintained on 1–1.5% halothane in O_2-enriched air via a face mask. Mice were not fasted before induction of anesthesia. Rectal temperature was maintained at approximately 36.5–37°C with a warming blanket and heating lamp during surgery and during the recovery period from anesthesia. Focal ischemia was produced by the intraluminal filament technique (Goto et al., 2002) by inserting a 6-0 nylon monofilament into the right internal carotid artery via an external carotid artery stump while the common carotid artery was occluded. The monofilament tip, which was blunted by heating and coated with cyanoacrylate glue, was advanced 6 mm from the bifurcation of the internal carotid and pterygopalatine artery. This procedure reduces laser-Doppler flow over lateral parietal cortex by approximately 80%. The neck incision was closed, anesthesia was discontinued, and the mouse was allowed to awaken and to be scored for neurologic deficit on a 0–4 point scale: 0 = normal motor function; 1 = flexion of torso and contralateral forelimb on tail lift; 2 = circling to the contralateral side but normal posture at rest; 3 = leaning to the contralateral side at rest; 4 = no spontaneous motor activity. Mice with scores of 0 or 1 were excluded from the study. Sham-operated animals underwent the same surgical procedure, but the suture was not advanced into the internal carotid artery. To establish reperfusion, the mouse was briefly anesthetized with halothane, the suture around the common carotid artery was untied, and the monofilament was withdrawn through the external carotid artery stump.

Behavioral outcome and infarct volume

Sensorimotor deficit and infarct volume were used to test if the chosen durations of 30, 60, and 120 minutes of transient MCAO produced gradations of injury in which the time course of AIF translocation with different severity of injury could be characterized. The cylinder test was performed 3 days after surgery to assess forelimb use and rotation asymmetry for each group, as previously described (Li et al., 2004). Briefly, the mouse was put into a transparent cylinder (9 cm diameter and 15 cm height), and forelimb use at first contact with the wall after rearing and during lateral exploration was recorded for a total of 20 movements during a maximum 10-minute test period. The observer was blinded to the experimental group. The asymmetry use score = (non-impaired forelimb movement − impaired forelimb movement) / (non-impaired forelimb movement + impaired forelimb movement + both movements), as described in rat (Schallert et al., 2000).

Infarct volume was determined 3 days after MCAO by image analysis of five 2-mm thick coronal sections stained with 1.5% solution of 2,3,5-triphenyltetrazolium chloride at 37°C for 30 minutes. The surface area of viable regions stained red and of nonviable regions that remained pale were integrated on both the anterior and posterior surfaces of each section. The average of the anterior and posterior viable surface areas was multiplied by the section thickness and summed to obtain the volume of viable tissue. Total infarct volume was expressed as a percentage of the contralateral hemisphere with correction for swelling (Swanson et al., 1990).

Subcellular fractionation and immunoblotting

Brains were rapidly harvested after decapitation, and subcellular fractionation was performed on the central portion of freshly isolated ischemic hemisphere after removing the anterior and posterior poles at approximately +2.2 mm and –4.9 mm from bregma (including cortex and striatum). In order to obtain sufficient protein in a highly purified nuclear pellet without detectable mitochondrial protein, relatively large tissue samples were required. The entire ipsilateral cortex and striatum between +2.2 and –4.9 mm from bregma were pooled from two mice. Immediately after harvesting and sectioning the fresh brain sample, the tissue was minced on a chilled metal plate, immediately placed into 10 ml of ice-cold buffer A (250 mM sucrose, 10 mM HEPES at pH 7.4, 1 mg/ml BSA, 0.5 mM EDTA, 0.5 mM EGTA), homogenized with

10 strokes of a handheld Teflon-glass homogenizer on ice, and then centrifuged at 2,000 g for 3 minutes at 4°C. To obtain the mitochondrial and cytosolic enriched fractions, the supernatant was transferred and further centrifuged for 8 minutes at 12,000 g, producing a cytosolic fraction in the supernatant (S2) that was stored at –80°C. The pellet was resuspended in buffer A, centrifuged at 12,000 g for 10 minutes, and the resulting pellet was further resuspended in 250 mM sucrose and centrifuged at 12,000 g for 10 minutes. The mitochondrial fraction in the final pellet was resuspended in 250 mM sucrose and stored at −80°C.

The nuclear-enriched fraction was obtained by ultracentrifugation with a sucrose gradient (Wang et al., 2004). The loose pellet from the first centrifugation was homogenized with a Dounce homogenizer in buffer A followed by suspension in 4 ml of buffer B (2 M sucrose, 5 mM Mg-acetate, 0.1 mM EDTA, 10 mM Tris-HCl at pH 8.0, 1 mM DTT). This suspension was placed in an ultracentrifuge tube on top of 4.4 ml of buffer B, followed by the addition of 2–3 ml of buffer C (320 mM sucrose, 3 mM $CaCl_2$, 2 mM Mg-acetate, 0.1mM EDTA, 10 mM Tris-HCl at pH 8.0, 1 mM DTT, 0.5% IGEPAL CA-630) for balance. The sample underwent ultracentrifugation at 30,000 g for 45 minutes at 4°C. The pellet containing the nuclear fraction was resuspended in glycerol storage buffer and stored at –80°C.

Western immunoblotting was performed by standard techniques on 4–12% Tris-glycine gels. Primary antibodies for 57 kDa AIF (rabbit polyclonal antibody, 1:2000 dilution) and the mitochondrial protein, manganese superoxide dismutase (MnSOD, 23 kDa, rabbit polyclonal antibody 1:10000 dilution), have been previously described (http://www.sciencemag.org/cgi/content/full/297/5579/259/DCI) (Yu et al 2002). A primary antibody for 12–15 kDa histones (sheep polyclonal antibody, dilution of 1:500, USBiological, Swampscott, MA) was used as a protein-loading control for the nuclear fraction. A rabbit polyclonal antibody, 42 kDa actin (dilution of 1:5000, Sigma, St. Louis, MO), was used as a protein-loading marker for the cytosolic fractions. The membranes were incubated in the primary antibodies over night at 4° C. Horseradish peroxidase-conjugated anti-rabbit (1:4000, BIO-RAD, Hercules, CA) and anti-sheep (1:5000 dilution, Pierce, Rockford, IL) secondary antibodies were incubated for 1 hour at room temperature and detected with ECL reagents. After detection, the films were quantified with Imagequant software (Molecular Dynamics, Sunnyvale, CA).

For time-course experiments with transient MCAO, each gel was designed to have a lane for sham-operated, 20-minute reperfusion, 6-hour reperfusion, and 24-hour reperfusion groups along with an AIF internal standard. This design permitted evaluation at early reperfusion, at a mid-range of reperfusion, when many neurons show ischemic morphologic changes, and at late reperfusion, when cell death is widespread throughout the maturing infarct. These time-points spanned the 1–8 hour range for AIF translocation reported by others (Ferrer et al., 2003;Plesnila et al., 2004;Zhao et al., 2004;Culmsee et al., 2005). Two hemispheres were used per lane. Separate experiments were performed for MCAO of 30, 60, or 120 minute-duration with 5 independent gels using tissue from 8 different mice for the 4 time-points represented on each gel. Thus, data for nuclear AIF time-course were generated from 40 mice for each MCAO duration. These experiments were designed to evaluate the time course at equivalent times of reperfusion rather than at equivalent times from the onset of transient MCAO. For the permanent MCAO experiment, lanes were allocated for sham-operated and 1-, 6-, and 24-hour permanent MCAO groups. Separate shams were used in each experiment.

For immunoblots of PAR, striatal samples were dissected from mice at 0 minutes, 20 minutes, 1 hour, 6 hours, or 24 hours of reperfusion. Samples were homogenized in 200 μl NuPAGE LDS Sample Buffer (NP0007, Invitrogen, Carlsbad, CA). Equal samples were loaded on 4–12% Tris-glycine gels. Western immunoblotting was performed by standard techniques. Primary antibody was rabbit anti-poly(ADPribose) polyclonal antibody LP96-10 to poly(ADP-

ribose) used at a dilution of 1:3000 (Affar et al., 1999). Anti-human β–III tubulin antibody (Chemicon, Temecula, CA) was used as a protein loading control.

For evaluating the role of nNOS in nuclear translocation of AIF, two experiments were performed. First, AIF in the nuclear fractions was compared between wildtype (WT) and $nNOS^{-/-}$ mice. Each gel had a brain nuclear fraction loaded from sham-operated WT and $nNOS^{-/-}$ mice and from WT and $nNOS^{-/-}$ mice subjected to 2 hours of MCAO and 24 hours of reperfusion. Comparisons between WT and $nNOS^{-/-}$ on the same gel were limited to the 24-hour reperfusion time point to permit adequate time for accumulation of AIF in the nucleus. In the second nNOS experiment, WT mice were treated with either vehicle (peanut oil; 16 μl/g, ip) or 7-NI (40 μg/g, ip) 30 minutes before MCAO or sham surgery. For each gel, the nuclear fraction was loaded from the brains of sham-operated mice 24 hours after treatment with vehicle or 7-NI and from the brains of vehicle- and 7-NI-treated mice after 2 hours of MCAO and 24 hours of reperfusion. To determine if inhibition of nNOS reduced the early formation of PAR, immunoblots for PAR were run on tissue from mice treated with vehicle or 7-NI.

Experimental design and statistical analysis

The intensity of the nuclear AIF bands was normalized by an internal standard consisting of an aliquot of the mitochondrial fraction from a single brain of a mouse that did not undergo surgery. The bands were not normalized by the sham value because this band had low and variable intensity, which could have been due to a small and variable amount of residual mitochondria in the nuclear fraction. Comparisons of immunoblot optical density among four lanes on the same gel were made by randomized block analysis of variance (ANOVA) with each gel as a blocking factor. If the *F*-value was significant, comparisons among the individual mean values were made by the Newman-Keuls multiple range test. A separate ANOVA was performed for 30, 60, and 120 minutes of MCAO and for permanent MCAO ($n = 5$ gels per experiment). For the $nNOS^{-/-}$ experiment, ANOVA was performed on 4 gels with four lanes loaded from WT and $nNOS^{-/-}$ mice after sham operation or MCAO. For the 7-NI experiment, ANOVA was performed on 6 gels with four lanes loaded from vehicle or 7-NI-treated mice after sham operation or MCAO. Infarct volume and forelimb asymmetry score were analyzed by one-way ANOVA and the Newman–Keuls multiple range test for *post hoc* comparisons of 30-minute MCAO ($n = 8$), 60-minute MCAO ($n = 9$), 120-minute MCAO ($n = 8$), and sham-operated ($n = 5$) groups. All data are expressed as mean ± SD. A significance level of $P < 0.05$ was used in all tests.

RESULTS

Effect of ischemic duration on AIF translocation

The antibody targeted against the 57 kDa-form of AIF primarily recognized the 57-kDa form in the mitochondrial subcellular fraction of mouse brain, although a minor 67-kDa band was also present (Figure 1A). A faint band at 57 kDa was present in the nuclear subcellular fraction of sham-operated mice. The intensity of this band increased in mice subjected to 30 minutes of MCAO followed by 24 hours of reperfusion. In five independent experiments, a significant increase in nuclear AIF immunoreactivity occurred at 24 hours of reperfusion (Figure 1B). However, no significant change in AIF in the nuclear fraction was observed at 20 minutes or 6 hours of reperfusion compared with the sham group. The adequacy of nuclear and mitochondrial separation was confirmed on every gel by the lack of immunoreactivity of the mitochondrial protein MnSOD and the presence of histones in every nuclear fraction.

When the duration of MCAO was extended to 60 minutes, a significant increase in nuclear AIF was detected at as early as 20 minutes of reperfusion (Figure 2A). The amount of AIF in

the nuclear fractions increased further after 6 and 24 hours of reperfusion. Because AIF binds to DNA and does not leave the nucleus, this progressive increase represents an accumulation of AIF in the nucleus. After 120 minutes of MCAO, no AIF translocation was found at 20 minutes of reperfusion, but translocation was prominent at 6 and 24 hours of reperfusion, with the 24-hour value significantly greater than the 6-hour value (Figure 2B).

No significant increase in nuclear AIF occurred with 1 hour of permanent MCAO (Figure 2C), in contrast to the increase seen with 20 minutes of reperfusion after 1 hour of MCAO. However, significant increases became evident by 6 hours of permanent MCAO, with further increases occurring at 24 hours of permanent MCAO.

Protein loading from the nuclear fraction was evaluated by measuring the density of the histone band. The coefficient of variation (100 SD/mean) of the histone band density among the four lanes (sham, 20 min or 1 h, 6 h, 24 h) ranged from 6–21% for the 20 gels in the four experimental groups, with an average of 12.7% and an SD of 4.2%. This percent variation was substantially less than the percent increases in the AIF band density. Moreover, repeated measures ANOVA of histone band density over the four time points did not reveal any significant change in histones over time in any of the four groups. Thus, the increase in AIF was not attributable to a bias in protein loading with time.

Mitochondrial and cytosol fractions were analyzed for AIF immunoreactivity after 60 minutes of MCAO. This ischemic duration was chosen because changes in nuclear AIF were most rapid after 60 minutes of MCAO. Immunoreactivity of AIF persisted in the mitochondrial fraction throughout 24 hours of reperfusion, but none was detected in the cytosol (Figure 3A). In five independent experiments, no significant change in mitochondrial AIF immunoreactivity was detected at 20 minutes, 6 hours, or 24 hours of reperfusion (Figure 3B), and no AIF was detected in the cytosol.

PAR time course

The time course of increased PAR immunoreactivity was evaluated on whole-cell tissue homogenates after 60 or 120 minutes of MCAO to determine if the increase in nuclear AIF coincided with an increase in PAR. After 60 minutes of MCAO, increased PAR bands were detected at 20 minutes through 24 hours of reperfusion (Figure 4). After 120 minutes of MCAO, the increase in PAR was significant at 20 minutes and 1 hour of reperfusion and subsided at 6 and 24 hours of reperfusion. An increase in PAR was also seen without reperfusion in some of the gels of both groups, but the increase was not as consistent as that seen at 20 minutes of reperfusion.

Sensorimotor deficit and infarct volume

To ensure that 30, 60, and 120 minutes of MCAO produced varying levels of injury, separate cohorts were survived for 3 days. When placed in a vertical cylinder, sham-operated mice reared up and explored the environment with equal use of both forelimbs. However, three days after 30, 60 or 120 minutes of MCAO, significant dose-dependent asymmetry in the use of the forelimbs occurred (Figure 5A), with values in each ischemic group significantly different from each other and from a sham-operated group. Likewise, 30, 60, and 120 minutes of MCAO produced graded increases in infarct volume that differed significantly among the three individual groups (Figure 5B). The forelimb use asymmetry score was highly correlated (r = 0.93) with infarct volume (Figure 5C).

Effect of nNOS on AIF translocation

In both WT and nNOS$^{-/-}$ sham-operated mice, only a faint amount of AIF was detected in the nuclear fraction (Figure 6A). After 2 hours of MCAO and 24 hours of reperfusion, the amount of AIF that accumulated in the nucleus increased in both WT and nNOS$^{-/-}$ brain tissue. However, the amount of nuclear AIF in the nNOS$^{-/-}$ brains was attenuated by 37%, compared to the WT brains (Figure 6C). In sham-operated WT mice treated with vehicle or 7-NI, the amount if AIF in the nuclear fraction was small (Figure 6B). Increased AIF accumulated in the nucleus after 2 hours of MCAO and 24 hours of reperfusion in both vehicle- and 7-NI-treated mice, but the amount in the 7-NI-treated group was reduced by 43% (Figure 6D). Treatment with 7-NI was also found to decrease PAR formation at 20 minutes of reperfusion after 2 hours of MCAO (Figure 7).

DISCUSSION

The major findings of this study are 1) that translocation of AIF to the nucleus was more rapid after 60 minutes of MCAO than after 30 or 120 minutes of MCAO or permanent MCAO, 2) that formation of PAR was rapid after 60 and 120 minutes of MCAO, and 3) inhibition or gene deletion of nNOS attenuated AIF translocation after MCAO.

In selecting the durations of transient MCAO, it was important to ensure that each duration produced a different degree of injury in order to determine how the time course of AIF translocation to the nucleus depended on the severity of the insult. Durations of 30, 60, and 120 minutes of MCAO with the filament technique in the mouse were found to produce both gradations of infarct volume and deficits in sensorimotor function, and these outcomes were highly correlated with each other.

After 30 minutes of MCAO, detection of nuclear AIF was delayed beyond 6 hours of reperfusion. The delayed increase in nuclear AIF at 24 hours is consistent with the observation that cell death occurs over a period of days, and even weeks, when focal ischemic duration is brief (Du et al., 1996;Katchanov et al., 2003). For example, infarct volume in rats subjected to 30 minutes of MCAO plus bilateral common carotid artery occlusion increased between 3 days and 14 days of reperfusion to a size equivalent to that seen with 90 minutes of ischemia (Du et al., 1996). We cannot exclude that differences in infarct volume seen at 3 days in the present study will diminish over a period of weeks. Although prolonged delays in neurodegeneration are presumed to have a major caspase-dependent component, the present results suggest that an AIF component, which is independent of caspases, may also participate.

A 30-minute duration of MCAO is known to produce energy depletion, ionic shifts, and transcellular water flux in a significant portion of the MCAO territory, but these changes are rapidly reversible upon reperfusion in much of this territory (Memezawa et al., 1992;Miyabe et al., 1996;Gido et al., 1997). However, when the duration of MCAO is extended to 60 minutes, a substantial amount of the MCA territory does not exhibit recovery of the water diffusion constant (Miyabe et al., 1996), thereby indicating persistent loss of transcellular ionic gradients during reperfusion. The increase in nuclear AIF seen at as early as 20 minutes of reperfusion after 60 minutes of MCAO implies that a significant portion of cells are rapidly undergoing irreversible cell death at this time. This finding with AIF immunoblotting is consistent with immunohistochemical detection of nuclear AIF in a small percent of cells by 1 hour of reperfusion and a larger increase that occurs by 4–8 hours of reperfusion after 45 minutes of MCAO in the mouse (Plesnila et al., 2004). Nuclear staining for AIF has also been reported in the rat brain at 4 hours of reperfusion after 1 hour of MCAO (Ferrer et al., 2003). An increase in the production of reactive oxygen species (ROS) leads to DNA damage and activation of PARP after MCAO (Zhang et al., 1994;Eliasson et al., 1999). In neuronal culture, activation

of PARP is linked to a signaling process that translocates AIF from the mitochondria to the nucleus (Yu et al., 2002;Wang et al., 2004). The rapid increase in PAR and the initiation of AIF translocation at 20 minutes of reperfusion after 60 minutes of MCAO is consistent with this interpretation.

When ischemic duration was extended to 120 minutes, AIF translocation was substantial at 6 hours, but was unchanged at 20 minutes of reperfusion. Secondary energy failure and associated ROS production would be expected to occur sooner during reperfusion, when the preceding ischemic duration is prolonged. Immunoblots demonstrated a 7-NI-sensitive increase in PAR by 20 minutes of reperfusion after 120 minutes of MCAO, thereby suggesting that NO-dependent DNA damage and consequent PARP activation were rapid with this ischemic duration. Others using immunohistochemical staining also reported a rapid increase in PAR immunoreactivity at 5 minutes of reperfusion after 120 minutes of MCAO in mouse (Endres et al., 1997). Thus the lack of an increase in nuclear AIF at 20 minutes of reperfusion after 120 minutes of MCAO, in contrast to the early increase in both AIF and PAR seen after 60 minutes of MCAO, was unexpected. Perhaps recovery of the redox potential of specific mitochondrial proteins, energy metabolism, and basic cell function may be required for rapid AIF translocation in concert with PARP activation, and that initial mitochondrial recovery may be sluggish after 120 minutes of ischemia in most of the cells. More widespread energy failure leading to early organelle dysfunction and necrosis may interfere with AIF translocation mechanisms. In addition, mechanisms independent of PARP activation may contribute to AIF translocation after severe ischemia.

It is of interest that PAR decreased between 20 minutes and 6 hours of reperfusion, while nuclear AIF was concurrently increasing. Because AIF remains bound within the nucleus long after translocation, the time course of AIF accumulation in the nucleus is expected to represent the time integral of the stimulus for translocation. Thus, the time-course data showing that increased PAR precedes or coincides with increased nuclear AIF are consistent with PARP activity providing a stimulus for AIF translocation. The decline in PAR after 1 hour of reperfusion is presumably related to the poly(ADP-ribose) glycohydrolase breakdown of PAR exceeding PAR formation by PARP.

With permanent MCAO, nuclear AIF increased at 6 hours and continued to accumulate at 24 hours of MCAO, which is consistent with the time-dependent cell death and the spread of infarction. Progressive increases in nuclear AIF have also been reported over 48 hours of permanent distal MCAO in the rat (Zhao et al., 2004). A recent study in mice reported progressive increases in nuclear AIF over the first 24 hours of permanent MCAO, and the increase observed at 4 hours was attenuated by an antioxidant (Lee et al., 2005). Thus, oxidant stress may also contribute to AIF translocation in permanent ischemia. Interestingly, no increase in nuclear AIF was observed in the present study with 1 hour of MCAO without reperfusion. Thus, the increase in nuclear AIF seen with 1 hour of MCAO plus 20 minutes of reperfusion indicates that reperfusion after moderate ischemic duration accelerates AIF translocation. Hence, if AIF is playing a critical role in neuronal cell death during ischemia-reperfusion, it will be optimal to block this pathway at the onset of reperfusion.

An increase in AIF in the cytosolic fraction and a decrease in the mitochondrial fraction have been reported by 8 hours of permanent ischemia (Zhao et al., 2004). However, in the present study, AIF was undetectable in the cytosolic fraction and was unchanged in the mitochondrial fraction. Methodological differences in subcellular fractionation or in the experimental model may account for the different findings. Our results imply that transit of AIF through the cytosol and uptake into the nucleus is rapid and that the amount of AIF that translocates is a small fraction of the mitochondrial pool.

Previous work has demonstrated that nNOS activation, PARP activation, and AIF translocation are causally linked in cell death elicited by N-methyl-D-aspartate excitotoxicity (Zhang et al., 1994;Wang et al., 2004). In experimental stroke, both nNOS and PARP activity were found to be increased (Eliasson et al., 1997;Endres et al., 1997;Eliasson et al., 1999;Goyagi et al., 2003), and gene deletion or pharmacologic inhibition of nNOS or PARP-1 reduced infarct volume (Huang et al., 1994;Eliasson et al., 1997;Endres et al., 1997;Goto et al., 2002). In addition, inhibition of PARP decreased AIF translocation, and knockdown of AIF in Harlequin mice reduced infarct volume after 45 minutes of MCAO (Culmsee et al., 2005). Direct exposure of cultured neurons to peroxynitrite stimulates AIF translocation (Zhang et al., 2002). After MCAO, $nNOS^{-/-}$ mice exhibit diminished peroxynitrite formation, as indicated by loss of nitrotyrosine immunoreactivity (Eliasson et al., 1999). Results from the present study demonstrating that the nNOS inhibitor 7-NI decreased PAR formation are consistent with previous work showing diminished PAR formation in $nNOS^{-/-}$ mice (Endres et al., 1998). Because comparable doses of 7-NI do not inhibit endothelial-dependent dilation to acetylcholine (Yoshida et al., 1994), the effects of 7-NI are considered to be selective for nNOS. A new finding of the present study is that $nNOS^{-/-}$ mice or WT mice treated with 7-NI each had reduced nuclear accumulation of AIF, thereby indicating that activation of nNOS contributes to AIF translocation. Taken together with previous work, these findings are consistent with the concept of a sequential linkage during ischemia-reperfusion in which activation of nNOS leads to increased formation of peroxynitrite, DNA damage, activation of PARP, and translocation of AIF. However, it should be noted that these studies were performed on male mice and that the role of nNOS and PARP-1 in injury from MCAO appears to be different in female mice (McCullough et al., 2005). Furthermore, 7-NI treatment of male WT mice or nNOS gene deletion in male mice attenuated, but did not eliminate, AIF translocation after MCAO. Thus, mechanisms independent of nNOS activation can also contribute to AIF translocation. Expression of inducible NOS is known to increase in the ischemic region over a period of 12–48 hours (Iadecola et al., 1995). Thus, it is possible that NO derived from inducible NOS contributes to residual AIF translocation seen at 24 hours of reperfusion after suppression of nNOS activity or expression.

In summary, the present results indicate that neuronally derived NO significantly contributes to the translocation of AIF from mitochondria to the nucleus. Moreover, the time course of AIF accumulation in the nucleus varies with the duration of focal cerebral ischemia. A moderate increase in ischemic duration from 30 minutes of MCAO to 60 minutes of MCAO markedly shortened the appearance of AIF in the nucleus from a time window of 6–24 hours to only 0–20 minutes of reperfusion. Furthermore, reperfusion accelerated the appearance of nuclear AIF after 60 minutes of MCAO compared to permanent MCAO, consistent with the possibility of early oxidant stress triggering the signaling pathways that stimulate AIF translocation. If this translocation initiates irreversible execution of cell death, then therapies may need to be directed at targeting this pathway to improve the clinical efficacy of clot dissolution by tissue plasminogen activator in ischemic stroke.

Acknowledgements

The authors wish to thank Ellen Gordes for technical assistance and Tzipora Sofare, MD, for editorial assistance.

This work was supported by a grant from the National Institute of Neurological Disorders and Stroke (PO1 NS39148) and by a postdoctoral fellowship award from the Mid-Atlantic Affiliate of the American Heart Association (#0425645U).

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Abbreviations

AIF
: apoptosis-inducing factor

ANOVA
: analysis of variance

MnSOD
: manganese superoxide dismutase

MCA
: middle cerebral artery

MCAO
: middle cerebral artery occlusion,

NO
: nitric oxide

nNOS
: neuronal nitric oxide synthase

nNOS$^{-/-}$
: neuronal nitric oxide synthase gene deletion

PAR
: poly(ADP-ribose) polymers

PARP
: poly(ADP-ribose) polymerase

WT
: wildtype

7-NI
: 7-nitroindazole

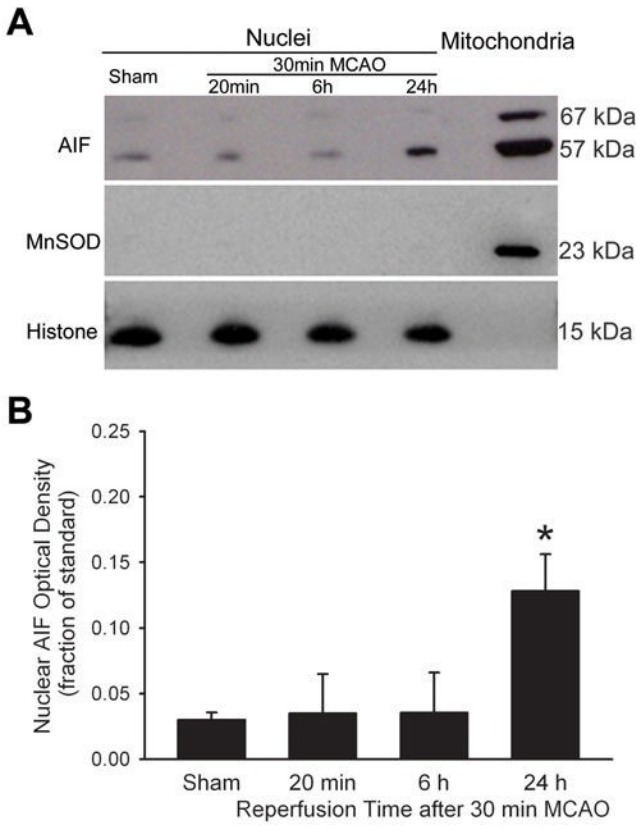

Figure 1.

(**A**) Western blot of AIF, MnSOD (mitochondrial protein marker), and histone (nuclear protein marker). Lanes 1–4 are nuclear fractions separated from cerebral hemispheres of sham-operated mice and from ischemic hemispheres of mice subjected to 30 minutes of MCAO and either 20 minutes, 6 hours, or 24 hours of reperfusion, with protein pooled from 2 hemispheres per lane. Lane 5 is a mitochondrial fraction from the hemisphere of a naïve mouse and was used as an internal standard for AIF on each gel. Note the absence of MnSOD in the nuclear fractions, indicating that there was no detectable contamination with mitochondria. (**B**) The optical density of AIF bands on each gel was normalized by the internal standard and presented as means ± SD for 5 gels. * $P < 0.05$ from sham group.

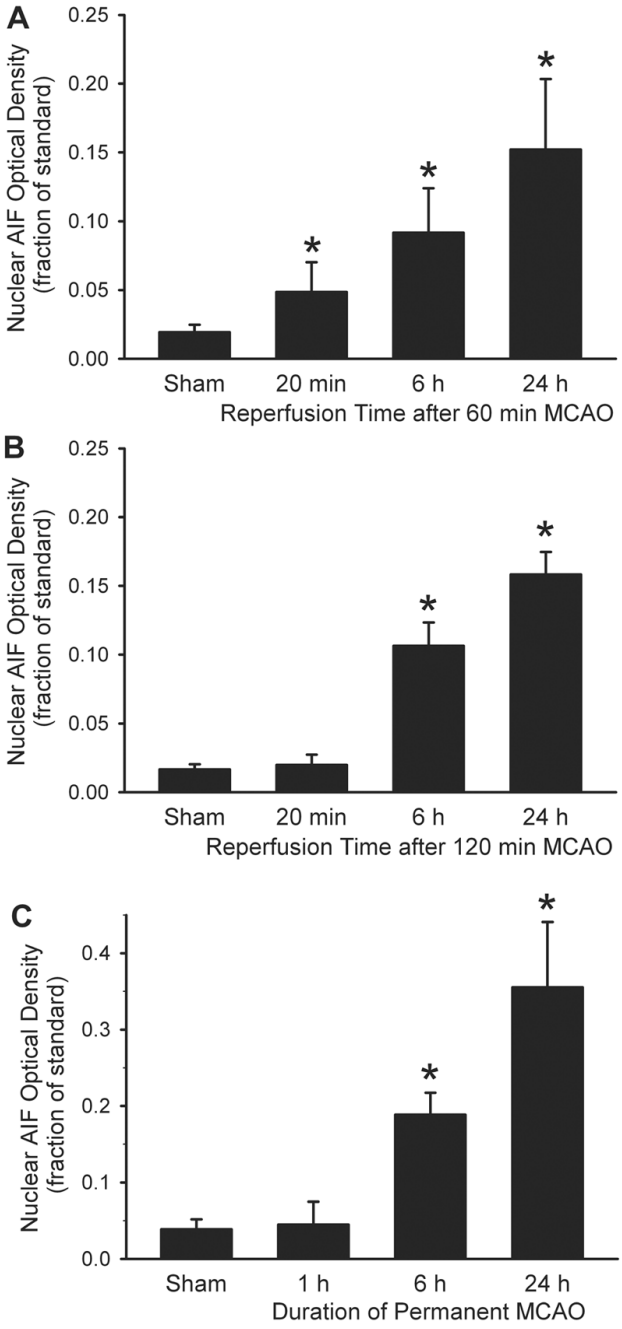

Figure 2.

The optical density of AIF in the nuclear fraction, normalized by the internal standard, is presented as means ± SD for 5 gels in each experiment: (**A**) sham and 20 minutes, 6 hours, and 24 hours of reperfusion after 60 minutes of MCAO; (**B**) sham and 20 minutes, 6 hours, and 24 hours of reperfusion after 120 minutes of MCAO; (**C**) sham and 1, 6, and 24 hours of permanent MCAO. * $P < 0.05$ from sham group.

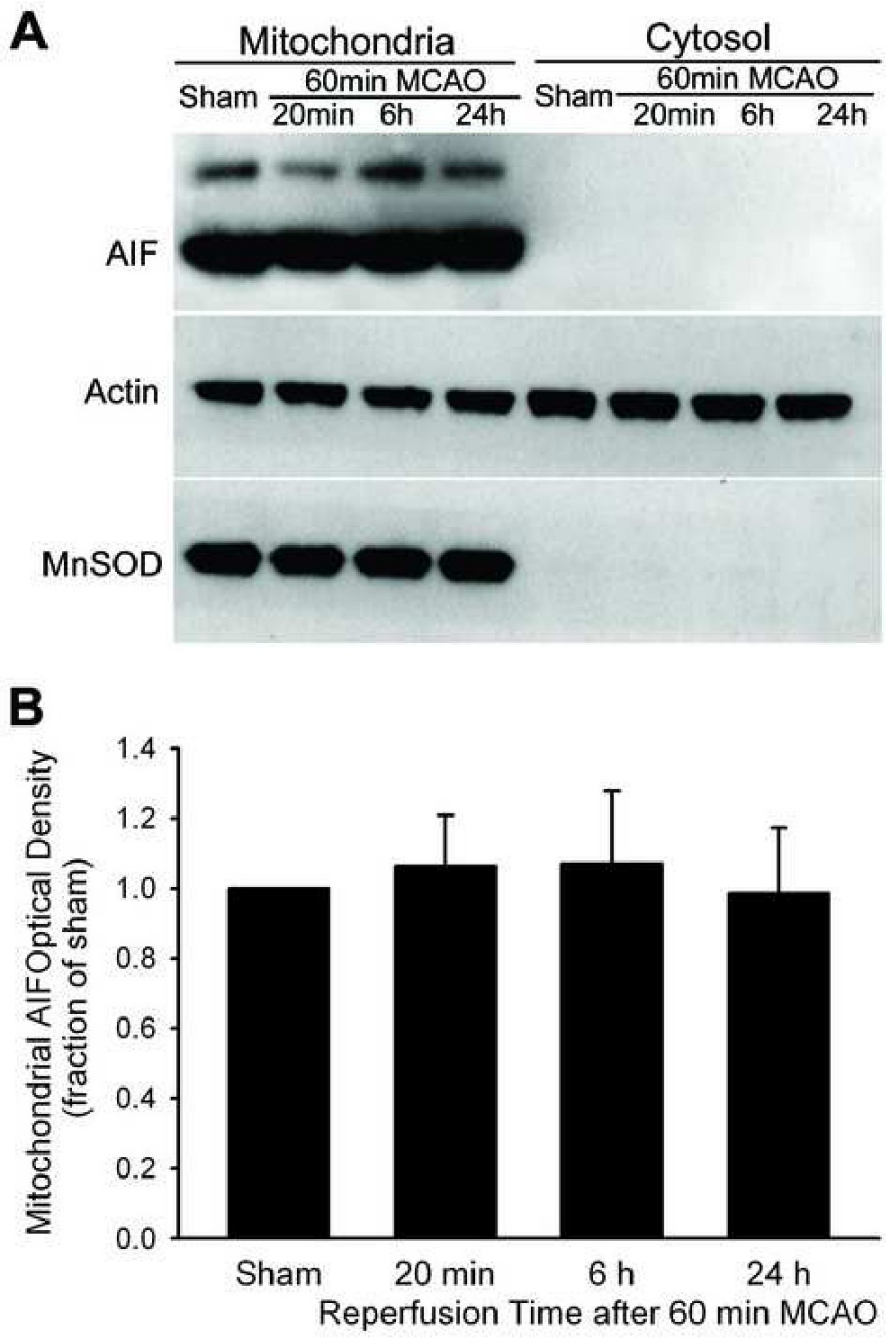

Figure 3.

(**A**) Western blot of AIF, MnSOD, and actin in mitochondrial (lanes 1–4) and cytosolic (lanes 5–8) fractions isolated from hemispheres of sham-operated mice and mice subjected to 60 minutes of MCAO and either 20 minutes, 6 hours, or 24 hours of reperfusion. AIF and MnSOD were enriched in mitochondrial fractions, but undetectable in cytosolic fractions in this and 4 other gels. (**B**) Optical density of AIF in mitochondrial fraction normalized by optical density of sham on each gel for 5 gels (means ± SD) did not show any significant change at 20 minutes, 6 hours, or 24 hours of reperfusion.

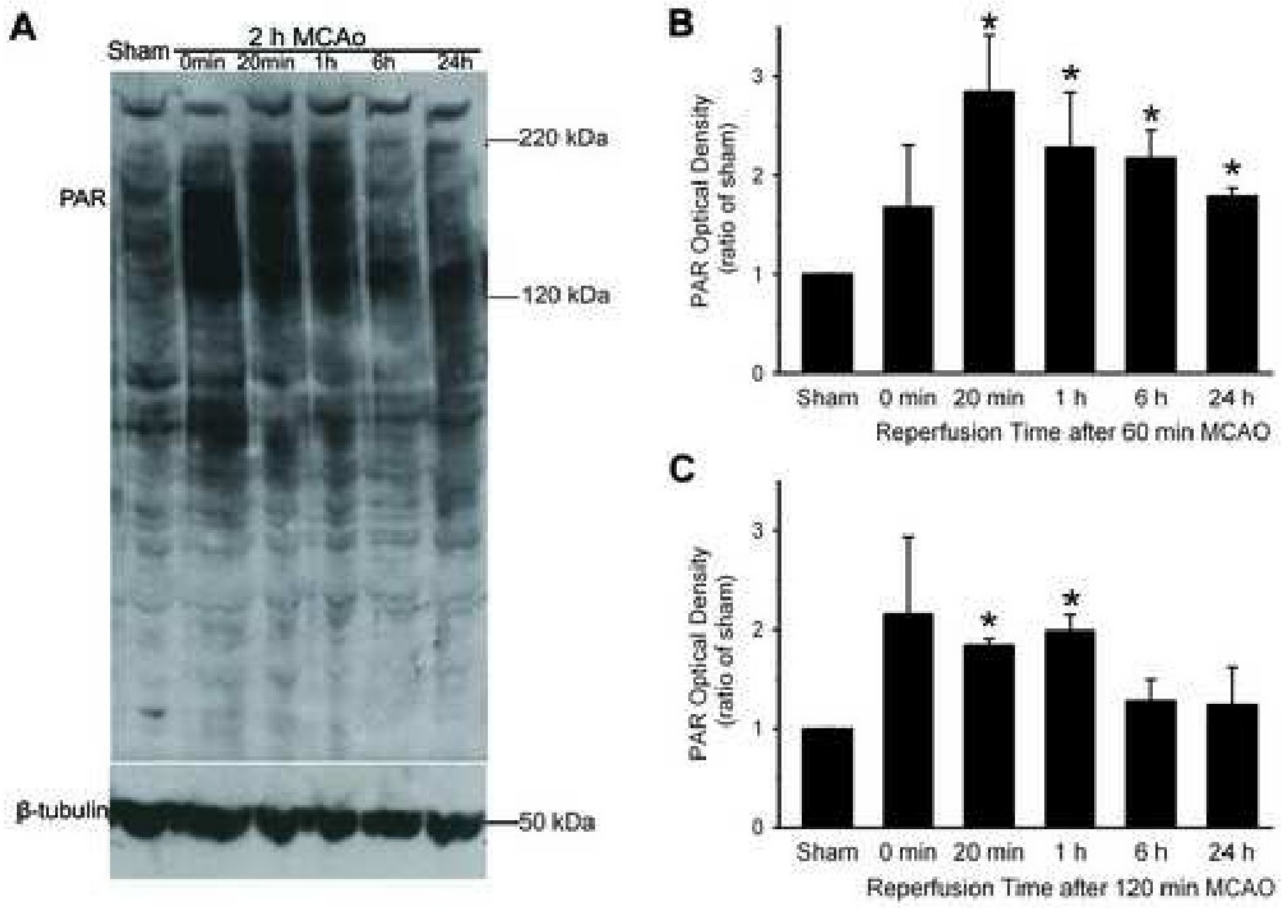

Figure 4.

A. Immunoblot of PAR from striatum of sham-operated mouse and of mice subjected to 2 hours of MCAO and either 0 minutes, 20 minutes, 1 hour, 6 hour, or 24 hours of reperfusion (with β-tubulin loading control bands). Note increase in PAR immunoreactivity of whole-cell homogenate over a large molecular weight range, including a band near 120 kDa, where poly (ADP-ribose) polymerization of 116 kDa PARP-1 is expected to migrate. Compared to sham, the increase in PAR bands was most prominent from 0 minutes through 1 hour of reperfusion, followed by a recovery at 6 and 24 hours. The optical density was quantified over a range from 110 to 220 kDa and expressed as a ratio of the sham on each gel. Results from 3 gels using tissue from mice subjected 60 minutes (**B**) and 120 minutes of MCAO (**C**) are presented as means ± SD. * $P < 0.05$ from sham group.

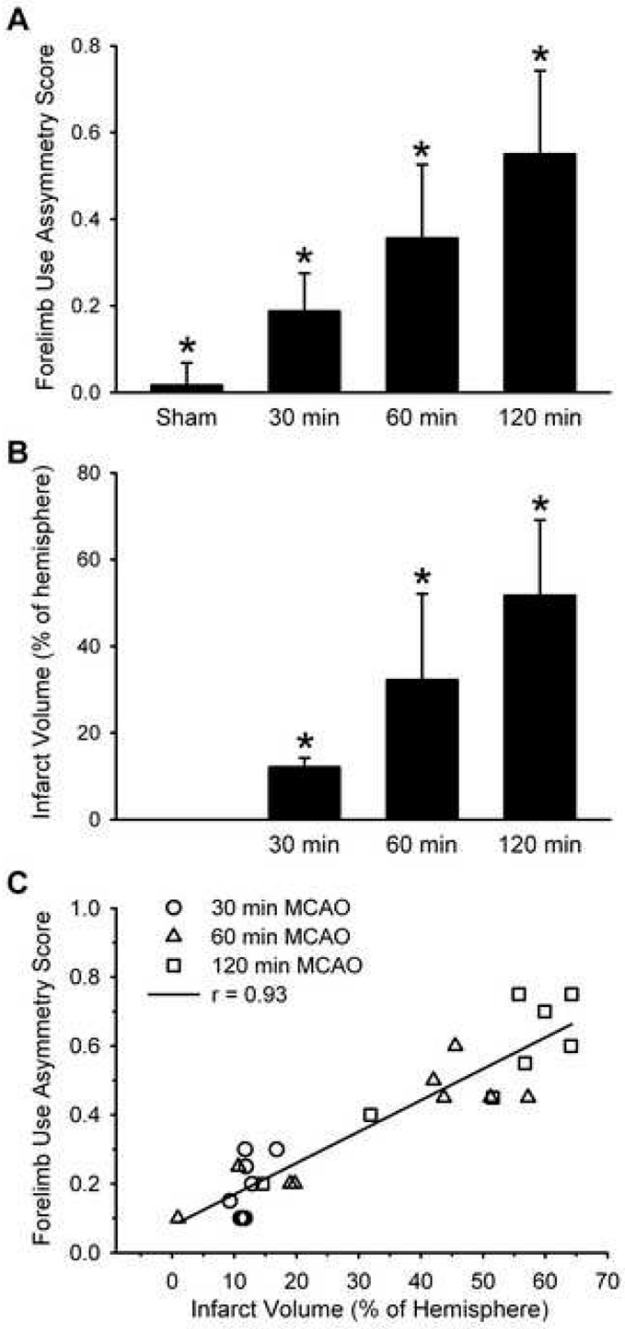

Figure 5.

Graded outcome (mean ± SD) at 3 days of reperfusion after transient focal ischemia for 30 minutes (n = 8), 60 minutes (n = 9), or 120 minutes (n = 8), and in a sham-operated group (n = 5). Forelimb use asymmetry score on the cylinder test (**A**) and infarct volume in cerebral hemisphere (**B**) in each group differed significantly from every other group by ANOVA and the Newman-Keuls test (* $P < 0.05$). (**C**) The forelimb use asymmetry score significantly correlated with infarct volume (r = 0.93).

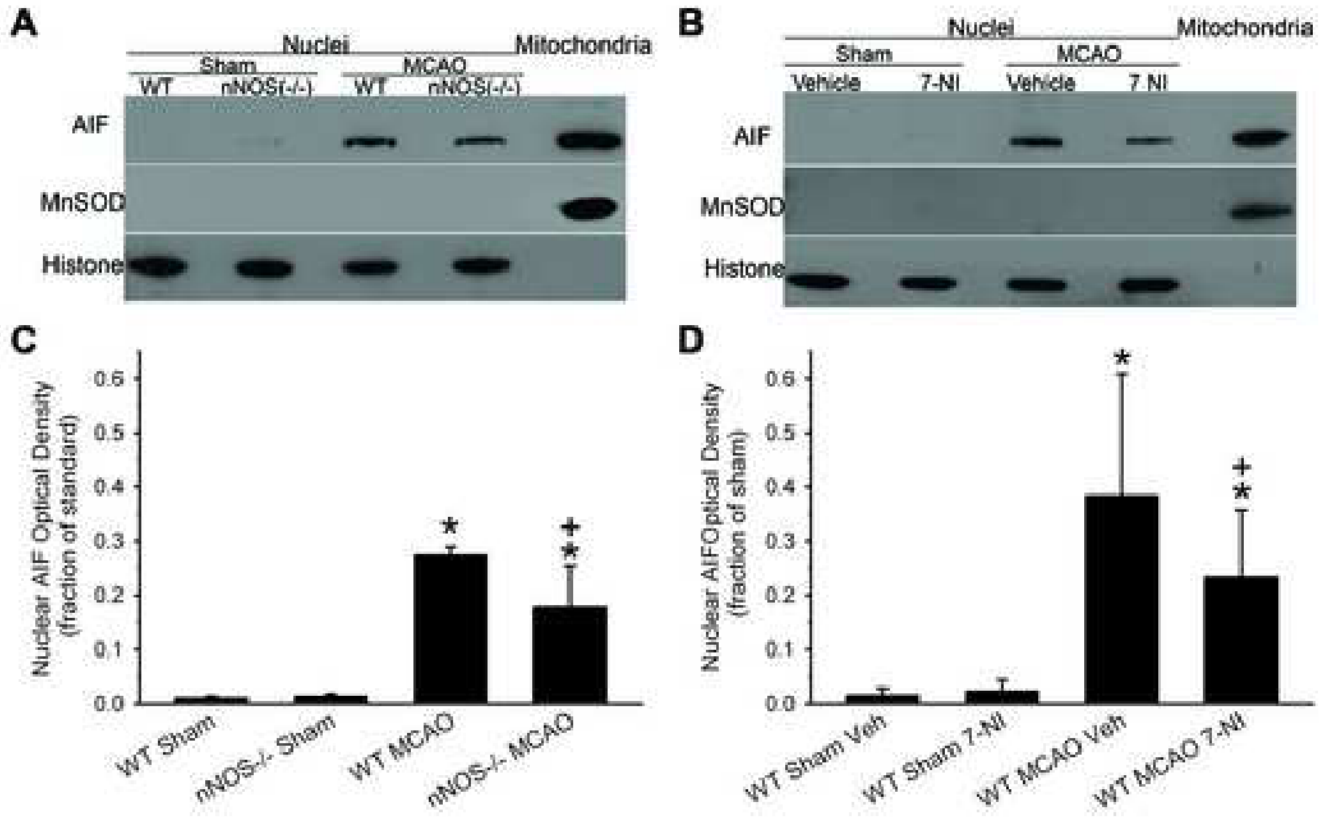

Figure 6.

(**A, B**) Western blots of AIF, MnSOD and histone. Lanes 1–4 are nuclear fractions separated from cerebral hemispheres of sham-operated mice and from ischemic hemispheres of mice subjected to 120 minutes of MCAO and 24 hours of reperfusion, with protein pooled from 2 hemispheres per lane. Lane 5 is a mitochondrial fraction from the hemisphere of a naïve mouse and was used as an internal standard for AIF on each gel. In **A**, nuclear fractions were loaded from sham-operated wildtype (WT) and neuronal nitric oxide null ($nNOS^{-/-}$) brains and from WT and $nNOS^{-/-}$ brains after MCAO. In **B**, nuclear fractions were loaded from sham-operated WT mice that were pretreated with vehicle or the nNOS inhibitor 7-nitroindazole (7-NI) and from mice pretreated with vehicle or 7-NI before MCAO. (**C, D**) The optical density of AIF bands on each gel was normalized by the internal standard and presented as means ± SD for 4 gels with WT and $nNOS^{-/-}$ mice (**C**) and for 6 gels with vehicle and 7-NI treatment (**D**). * $P < 0.05$ from respective sham group; + $P < 0.05$ from WT-MCAO group in **C** or from vehicle-MCAO group in **D**.

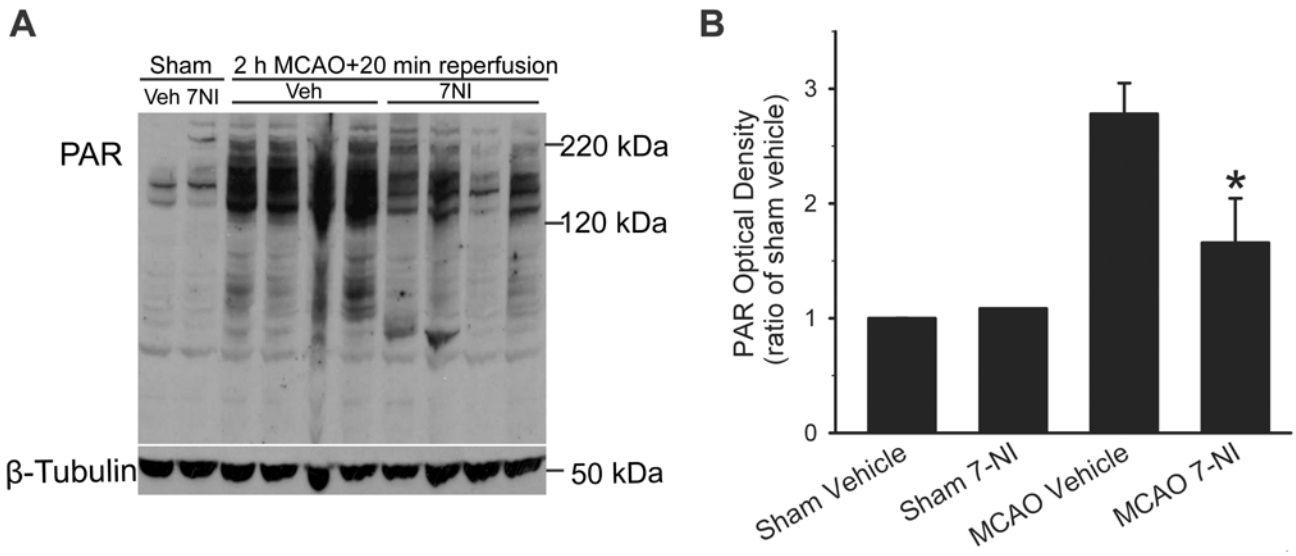

Figure 7.

A. Immunoblot of PAR from sham-operated mice treated with vehicle (Veh) or 7-nitroindazole (7-NI) and from mice subjected to 2 hours of middle cerebral artery occlusion (MCAO) and 20 minutes of reperfusion after treatment with vehicle or 7-NI. β-Tubulin was used as a loading control. **B**. Quantification of PAR optical density bands between 120–220 kDa were normalized by the sham vehicle value and expressed as mean ± SD. * $P < 0.05$ between MCAO vehicle ($n = 4$) and MCAO 7-NI ($n = 4$) groups.