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Mild hypothermia diminishes oxidative DNA damage and pro-death signaling events after cerebral ischemia: a mechanism for neuroprotection

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1. ABSTRACT

Mild hypothermia, applied either during or soon after cerebral ischemia, has been shown to confer robust neuroprotection against brain injury in experimental stroke and in patients recovering from cardiac arrest. However, the mechanism underlying hypothermic neuroprotection is not completely understood. In this study, the effect of mild hypothermia on the induction of oxidative DNA damage, an early harmful event during post-ischemic reperfusion that triggers both necrotic and apoptotic cell death in the brain, was studied using the rat model of middle cerebral artery occlusion (MCAO) and reperfusion. Rats were subjected to 2-hr MCAO and reperfusion of various durations up to 3 days. Selective brain hypothermia (33^{o}C) was induced at the onset of ischemia and terminated at the beginning of reperfusion, and this significantly decreased infarct volume 72 hr later. Correlated with this protective effect, intraischemic mild hypothermia markedly attenuated the nuclear accumulations of several oxidative DNA lesions, including 8-oxodG, AP sites, and DNA single-strand breaks, after 2-hr MCAO. Consequently, harmful DNA damage-dependent signaling events, including NAD depletion, p53 activation, and mitochondrial translocation of PUMA and NOXA, were reduced during post-ischemic reperfusion in hypothermia-treated brains. These results suggest that the attenuation of oxidative DNA damage and DNA damage-triggered pro-death signaling events may be an important mechanism underlying the neuroprotective effect of mild hypothermia against ischemic brain injury.

2. INTRODUCTION

Induced brain hypothermia is by far the most effective neuroprotective intervention against experimental stroke. In animal models of focal cerebral ischemia and reperfusion, a reduction in brain temperature of as little as 3-5^{o}C (mild hypothermia) during or immediately after ischemia is sufficient to remarkably reduce infarct volume and improve long-term functional outcomes. Despite the reproducible demonstration of hypothermic neuroprotection by laboratories and the current active pursuit of clinical trials of hypothermic therapy in stroke patients, however, the cellular and molecular mechanisms underlying hypothermic neuroprotection are not fully understood. A number of mechanisms have been proposed for mild hypothermia, including preservation of cellular energy stores, attenuation of release of excitatory amino acids (1), decreased formation of oxygen or nitrogen free radicals (2), reduced damage to the blood-brain barrier, and inhibition of post-ischemic inflammation (3-5).

Oxidative damage to genomic DNA is a critical early event that contributes to neuronal cell death following cerebral ischemia (6-8). Several types of highly cytotoxic DNA lesions, such as apurinic/apyrimidinic abasic site (AP site) lesions and single-strand breaks (SSB), are markedly induced in neurons as early as minutes after transient cerebral ischemia (6, 8-10). These oxidative lesions, when unrepaired promptly, may potently trigger various cell-killing signaling pathways, leading to necrosis or apoptosis in the ischemic brain (reviewed in (11)). Several studies have demonstrated an excellent spatial correlation between accumulation of oxidative DNA lesions and irreversible DNA fragmentation and cell death in different animal models of cerebral ischemia and reperfusion (9, 12-14). Thus, limiting oxidative DNA damage during early stages of ischemic injury and, consequently, prevention of DNA damage-induced intracellular pro-death signaling events may be an important mechanism underlying the neuroprotective effect conferred by antioxidant and related therapeutic interventions.

In the present study, we have investigated the effect of intra-ischemic mild hypothermia on post-ischemic induction of oxidative DNA damage using the rat model of transient focal ischemia. Selective brain cooling was achieved during ischemia while the body temperature was maintained in the normal range. The study aimed to test the hypothesis that mild hypothermia may protect against ischemic brain injury by limiting the early induction of oxidative DNA damage and attenuating DNA damage-specific pro-death signaling pathways.

3. MATERIALS AND METHODS

3.1. Animal model

All animal procedures were performed using protocols approved by the Animal Care Committee at the University of Pittsburgh and in accordance with the principles outlined in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. Temporary focal ischemia was induced in male Sprague-Dawley rats weighing 275-310 g using the intraluminal vascular occlusion method as previously described (15). To ensure the induction of ischemia by MCA occlusion, regional cerebral blood flow (rCBF) was monitored in two groups of rats (n = 8 per group) using laser Doppler flowmetry (LDF, PeriFlux System 5000, Perimed, Stockholm, Sweden) in the following coordinates: 0.5 mm anterior and 5.0 mm lateral from the bregma.

To induce hypothermia in the brain, a plastic bag containing powdered ice was placed directly on the rat skull and maintained with the close monitoring of brain temperature. Both brain (right caudate-putamen) and rectal temperatures were monitored throughout the experiment. Brain hypothermia was induced at the beginning of MCAO and withdrawn at the onset of reperfusion. Rectal temperature was kept in the range of 36.5-37.3^{o}C throughout the experiment using a temperature-regulated heating lamp and heating pad.

3.2. Measurement of infarct volume

Infarct volume was determined at 72 hr after 2-hr MCAO using the MCID image analysis system as previously described (15). Twenty-micrometer-thick serial coronal sections were obtained every 0.4 mm between the levels of +5.0 and –5.0 mm (anterior-posterior) from the bregma and stained with cresyl violet. Animals that showed a massive hematoma in the brain or no infarction in the brain were omitted from further histological analysis.

3.3. Measurement of 8-OhdG content using HPLC-EC detection

The content of 8-hydroxyl-2'-deoxyguanosine (8-oxodG) was measured in nuclear DNA extracts from brain tissues using high-performance liquid chromatography with electrochemical (HPLC-EC) detection (10). Rats were sacrificed at 0, 0.25, 0.5, 2, 8, 24 and 72 hr after 2 hr of focal ischemia, with or without hypothermic treatment (n = 6-8 per time point). The brains were rapidly removed, and the parietal cortices and caudate-putamina were separately dissected and stored at -80°C. The methods for nuclear DNA extraction and digestion were as previously described (10). Isocratic analysis was carried out on a CoulArray system (Model 5600) equipped with a dual piston (Model 580) and a PEEK pulse damper (ESA, Inc., Chelmsford, MA). The analysis was performed using two coulometer array cell columns. The data were acquired and analyzed using the CoulArray software, and expressed as the number of 8-OHdG in 10^5 2-dG determined in the same sample.

3.4. Quantitative measurement of AP sites in nuclear DNA

Nuclear DNA isolated from ischemic and sham brain tissues (n = 6 per time point) were subjected to quantitative measurement of AP sites using the calorimetric assay previously described (16). A biotin-labeled reagent specific for the aldehyde group in the ring-open form of AP site, designated as Aldehyde Reactive Probe (ARP), was used for the detection of AP sites (Dojindo Molecular Technologies, Gaithersburg, MD). ARP specifically binds to AP sites in isolated genomic DNA, and the biotin molecule in ARP can then be detected calorimetrically using a streptavidin/biotin complex conjugated to horseradish peroxidase. All ARP assays were performed in

triplicate, and the means were calculated. The data, expressed as the number of AP sites per 10^5 nucleotides, were calculated based on the linear calibration curve generated for each experiment using ARP-DNA standard solutions.

3.5. Detection of DNA single-strand breaks

The DNA polymerase I-mediated biotin-dATP nick-translation (PANT) assay was performed as previously described (8) on fresh-frozen sections from brains subjected to 2 hr of MCA occlusion at 0.5, 2, 4, 8, 24 and 72 hr of reperfusion, with or without hypothermic treatment (n = 6 per time point). This assay utilizes a two-step double-staining procedure that allows the detection of DNA single-strand breaks with either a 3'OH or a 3' blocked group. For the first step, sections were incubated in a moist-air chamber at 37°C for 90 min with the PANT reaction mixture containing 5 mM MgCI2; 10 mM 2-mercaptoethanol; 20 µg/ml bovine serum albumin; dGTP, dCTP, and dTTP at 30 µM each; 29 µM biotinylated dATP; 1 µM dATP; and 40 U/ml Escherichia coli DNA polymerase I (Sigma) in PBS (pH 7.4). The biotin-dATP incorporated in DNA was detected using Texas Red Avidin D (cell sorting grade; Vector Laboratories, Burlingame, CA). For the second step, the sections were incubated for 2 hr in a reaction mixture containing Endonuclease IV (100 U/ml), which recognized and cleaved the 3' blocking group in DNA strand breaks, thus generating 3'OH. This was followed by a second incubation in the PANT reaction mixture, and then the biotin-dATP incorporated in DNA was detected using FITC Avidin D. PANT-positive cells were quantified using a computerized scanning program (MCID, St. Catharines, Ontario, Canada) as described previously (8).

3.6. Determination of NAD content in rat brain

Brain NAD content was quantitatively measured in the cortex and caudate-putamina at 2, 8, and 24 hr after 2 hr of MCAO with or without hypothermic treatment (n = 6 per group). The *in situ* freezing technique (14) was used in this study to minimize the changes of labile cerebral metabolites during decapitation and brain dissecting procedures. In brief, the rats were anesthetized with isoflurane, intubated and ventilated with 1.5% isoflurane in a mixture of 68.5% N_2O and 30% O_2. A skin incision was made in the midline to expose the skull, and a plastic funnel with a bottom diameter of 15 mm was fitted into the skin incision and secured by pulling the skin up around the funnel with sutures. Brain freezing was achieved by liquid N_2 through the funnel, which continued for 3 min; thereafter the respiratory tubing was disconnected and the whole head was immersed in liquid N_2. Dissection of brains was performed at -22°C, and approximately 50 mg of brain tissues was used for NAD measurement. Brain tissue was manually homogenized in 400 µl of 0.5M $HClO_4$ at 4°C and centrifuged (3000 x*g* for 15 min, -2°C). The pellet was used for protein determination and the supernatant was neutralized with 800 µl of 2M KOH/0.2M K_2HPO_4-KH_2PO_4. NAD content in the supernatant was measured using an enzymatic cycling method in which NAD is converted to NADH by alcohol dehydrogenase. NADH reduces thiazolyl blue through intermediation of phenazine methosulfate to the purple formazan, and the absorbance was measured at 556 nm using a spectrophotometer (Beckman DU 7400). The data were expressed as percentage changes over sham controls.

3.7. Immunohistochemistry

To detect the cellular poly(ADP-ribose)ation after ischemia, immunohistochemistry was performed using fresh frozen brain sections obtained at 2 and 8 hr after 2 hr of MCAO, with or without hypothermic treatment. Standard protocols were used for immunohistochemistry. The primary antibody was the rabbit anti-poly(ADP-ribose) polyclonal antibody (BIOMOL Research Laboratories, Plymouth Meeting, PA), used at a dilution of 1:100, followed by the biotinylated goat anti-rabbit IgG secondary antibody (1:1000) and the avidin-biotin-peroxidase method. Alternate sections were immunoreacted without the primary antibody as a control.

3.8. Statistical analysis

The statistical significance between groups was determined with analysis of variance (ANOVA). *Post hoc* testing used the Bonferroni *t* test, and $p < 0.05$ was accepted as statistically significant. All values are expressed as mean ± SD.

4. RESULTS

4.1. Mild hypothermia reduces ischemic infarct volume

The brain cooling method employed in the present study reduced brain temperature to the desirable range (34-32°C) within 15 min of the onset (Figure 1A), while body temperature was kept in the normal range throughout the experiment. There were no statistically significant differences in the physiologic variables (blood pressure, blood glucose, and blood gases) between the hypothermic and normothermic groups before, during or after MCAO (data not shown). Regional cortical blood flow measured using LDF during and after MCAO showed no significant differences between the hypothermic and normothermic groups (Figure 1B).

The effect of mild hypothermia on the infarct volume measured at 72 hr after 2 hr of MCAO is illustrated in Figure 1C-D. Intra-ischemic hypothermia had a robust neuroprotective effect on ischemic injury, reducing the total infarct volume by approximately 72%. The neuroprotection was manifested in both cortex and striatum, reducing infarct volume by ~80% and ~50%, respectively.

4.2. Mild hypothermia attenuates the induction of oxidative DNA base damage

To investigate the effect of mild hypothermia on the induction profiles of oxidative DNA base damage after severe focal ischemia and reperfusion, the frontal/parietal cortices and caudate-putamina were dissected separately at various time points after 2 hr of MCAO with or without hypothermic treatment (n = 6-8 per group), and then subjected to quantitative measurement for nuclear contents of 8-oxodG and AP site lesions, respectively. Figure 2 illustrates the profiles of 8-oxodG induction in cortex and caudate during post-ischemic reperfusion. In normothermia rats, the levels of 8-oxodG were significantly increased in

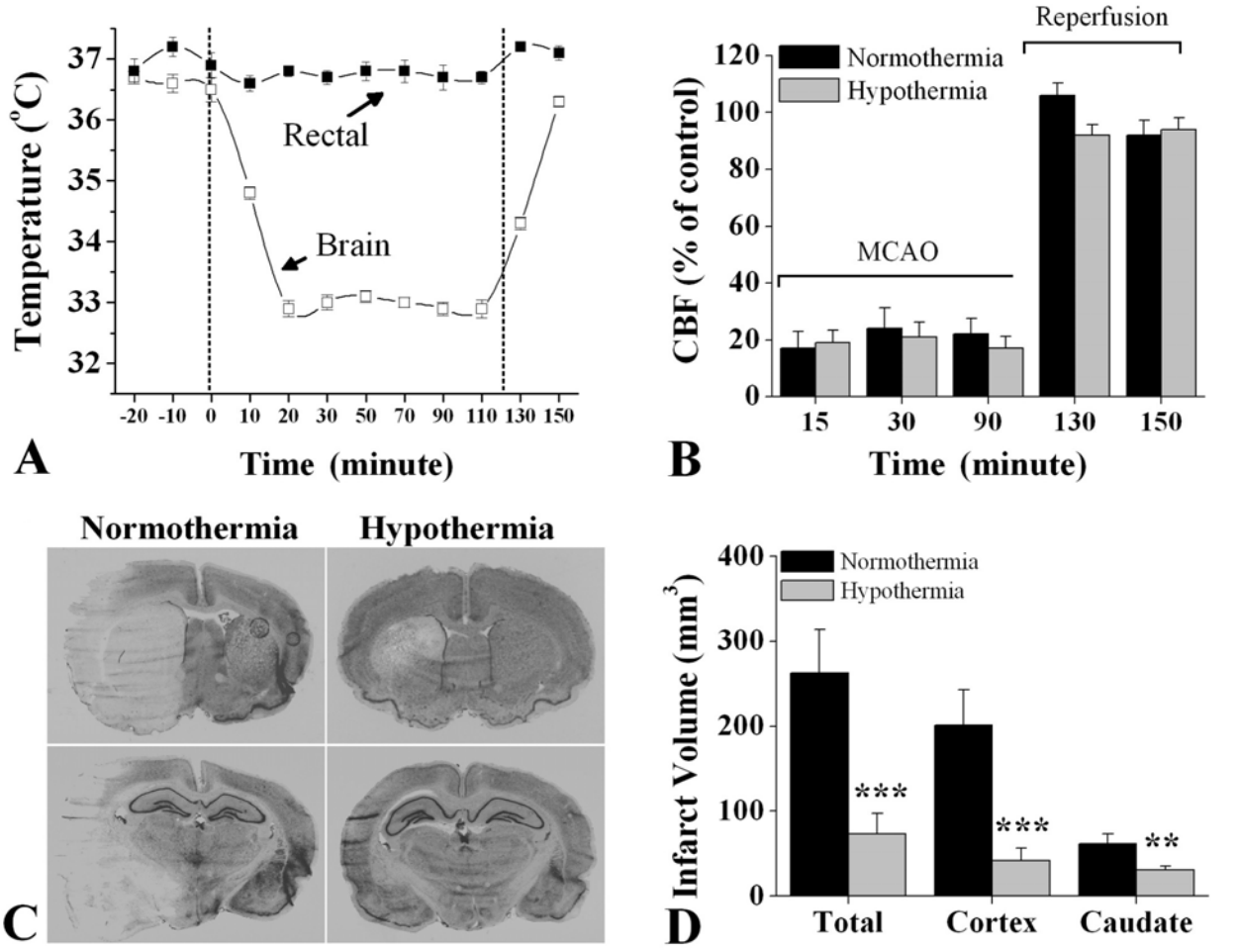

Figure 1. Verification of the neuroprotective effect of mild hypothermia in the rat MCAO model. A, brain and rectal temperature during and after MCAO in hypothermia-treated rats. Brain hypothermia was initiated at the onset of MCAO and then withdrawn at the beginning of reperfusion, whereas rectal temperature was maintained in the normal range throughout the experiment. B, changes in cortical blood flow, as determined using laser-Doppler flowmetry, are not different between normothermic and hypothermic brains during or after ischemia (n = 8 per group). C, representative cresyl violet-stained sections showing decreased infarct size in a hypothermia-treated brain. D, decreased infarct volume (total, cortex, and caudate-putamen) after 2-hr MCAO and 72-hr reperfusion in hypothermia-treated brains. ** $p < 0.01$, ***$p < 0.001$ *versus* normothermic controls.

the cortex and caudate within 15 min of post-ischemic reperfusion, and persistently elevated throughout the remainder of reperfusion (0.5-72 hr) in the cortex (4-7-fold) and caudate (8-12-fold). Hypothermic treatment had a remarkable inhibitory effect on 8-oxodG induction in the brain after MCAO. In the cortex, hypothermia completely abolished the increase in the levels of 8-oxodG; in the caudate, hypothermia significantly, however not completely, reduced the levels of 8-oxodG.

Figure 3 shows the effect of hypothermic treatment on the induction of AP site lesions in the brain after ischemia. The induction profiles of AP site in normothermia rats after ischemia were similar to that of 8-oxodG. A 2-fold increase in the levels of AP site was seen in the cortex as early as 0 hr of reperfusion, and massive increases of AP site were detected in both cortex (6-9-fold) and caudate (4-6-fold) at 0.5 hr of reperfusion and thereafter. In contrast, in hypothermia-treated rats, the increases in AP site were completely abolished in the cortex and significantly, but not completely, diminished in the caudate.

These results demonstrate that intra-ischemic mild hypothermia diminished the nuclear accumulation of 8-oxodG and AP site lesions in the cortex and caudate after focal ischemia and reperfusion, and this effect of hypothermia correlates with its neuroprotective effect in these two brain regions.

4.3. Mild hypothermia attenuates the induction of DNA strand breaks

Single-strand breaks (SSB) are a form of oxidative DNA damage that may contribute to ischemic

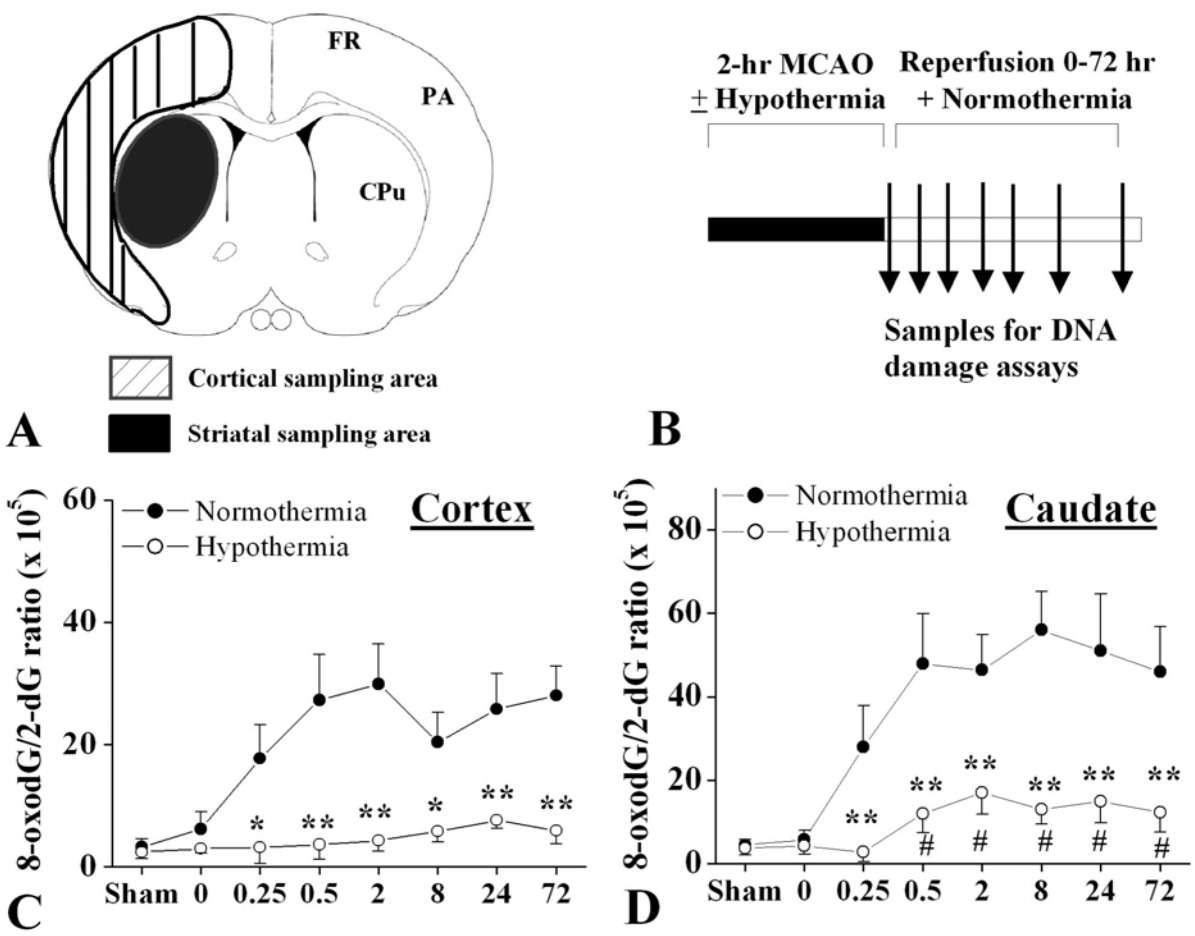

Figure 2. Intraischemic hypothermia attenuates the induction of 8-oxodG in the brain following focal ischemia and reperfusion. A, schematic diagram depicts regions from which tissues were sampled for DNA damage assays. FR, frontal cortex; PA, parietal cortex; Cpu, caudate-putamen. B, schematic diagram illustrates the protocol of tissue sampling for DNA damage assays in the present study. C-D, induction of 8-oxodG in the cortex and caudate-putamen after 2 hr of focal ischemia, with or without hypothermia treatment during MCAO. *p < 0.05; **p < 0.01 *versus* normothermia controls; #p < 0.05 *versus* sham-operated non-ischemic animals (n = 6-8 per time point).

cell death by activating the PARP-1-dependent signaling pathway (8, 10). In this study, the effect of hypothermic treatment on the cortical induction of SSB after 2-hr focal ischemia was investigated. The PANT assay was performed to detect SSB with either the 3'OH or 3' blocking group, and the results are summarized in Figure 4. In normothermia rats, both forms of SSB were markedly increased during post-ischemic reperfusion. In every time point examined, the number of cells containing the 3'blocking group was greater than that of the cells containing 3'OH. These results are consistent with the notion that, after severe focal ischemia, the repair process for AP site or SSB may be compromised, thus limiting the conversion of 3' blocking group to 3'OH. In contrast, both forms of SSB were significantly attenuated after ischemia in hypothermia-treated brains. Moreover, most of the remaining SSB cells in the hypothermia-treated brains contained the 3'OH instead of the 3'blocking group, suggesting that active DNA repair might be occurring in those cells.

4.4. Mild hypothermia attenuates NAD depletion induced by DNA damage

PARP-1 activation and NAD depletion is a well-characterized signaling pathway that mediates DNA damage-induced neuronal necrosis (17, 18). Since we found that hypothermic treatment diminished the accumulation of oxidative DNA lesions in the brain, we hypothesized that DNA damage-induced cellular signaling would be decreased in hypothermia-treated brains after ischemia. Therefore, two signaling events, poly(ADP-ribose)polymer formation and NAD depletion, which are direct consequences of PARP-1 activation, were investigated in brains after 2-hr focal ischemia with or without hypothermic treatment. Three different brain regions, including the caudate-putamen, the cortical region in relevance to the infarct core (ischemic core), and the cortical region in relevance to the infarct border (ischemic border), were selected for NAD measurement (Figure 5A). As expected, NAD contents were significantly decreased in the caudate-putamen and cortical ischemic core regions after ischemia

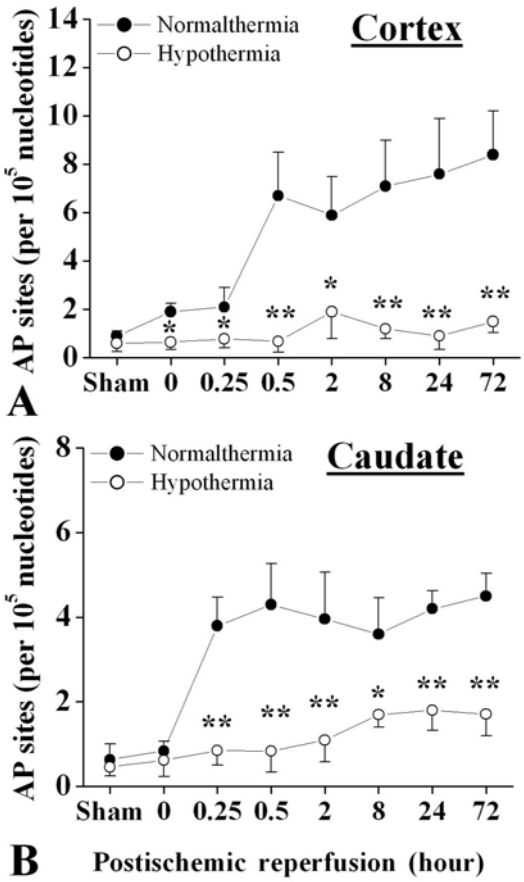

Figure 3. Intraischemic hypothermia attenuates the induction of AP site in the brain following focal ischemia and reperfusion. Induction of AP sites in the cortex (A) and caudate-putamen (B) after 2-hr focal ischemia, with or without hypothermia treatment during MCAO. *p < 0.05; **p < 0.01 *versus* normothermia controls; #p < 0.05 *versus* sham-operated non-ischemic animals (n = 6 per time point).

in a time-dependent manner (Figure 5C-D), whereas the levels were either unchanged (2 and 8 hr after ischemia) or increased (24 hr) in the ischemic border zone (Figure 5B). Intra-ischemic hypothermic treatment completely restored the NAD levels in the cortex and partially restored the levels in the caudate-putamen. Consistent with the NAD results, markedly increased immunoreactivity for poly(ADP-ribose)polymer was observed in ischemic cortex (the cortical ischemic core) and the caudate-putamen at 2 and 8 hr after 2-hr MCAO (Figure 5E), and morphological examination indicated that most poly(ADP-ribose)polymer-positive cells were neurons. The intensity of poly(ADP-ribose)polymer immunoreactivity was markedly decreased in brain that received hypothermic treatment during ischemia.

4.5. Mild hypothermia attenuates p53-related pro-apoptotic signaling events

Activation of the nuclear protein p53 is an important signaling event that contributes to cell death after ischemia. Accordingly, the phosphorylation of p53 (p-p53, Ser46), an indicator of p53 activation, was examined in cortical protein extracts at 2, 8, and 24 hr after 2-hr ischemia with or without hypothermic treatment. Increases in p-p53 were detected in the cortical ischemic border zone (Figure 6A), but not in the ischemic core regions (data not shown), after MCAO. Hypothermic treatment completely abolished the increases of p-p53 in the infarct border zone. Subsequently, we examined the expression of PUMA and NOXA, the two pro-apoptotic proteins downstream of the p53 signaling pathway, in the cortical ischemic border zone after MCAO with or without hypothermic treatment. PUMA and NOXA were both subjected to increased expression (Figure 6B) and mitochondrial translocation (Figure 6C) after MCAO in normothermic brains; these changes were completely abolished in hypothermia-treated brains.

5. DISCUSSION

The remarkable neuroprotective effect of mild hypothermia against ischemic brain injury is likely attributed to its broad inhibitory actions on a variety of harmful cellular processes induced by cerebral ischemia. Among the proposed key mechanisms underlying hypothermic neuroprotection is the blockage of intracellular signaling events that initiate the cell death cascade. In this study, using an established rat model of middle cerebral artery occlusion and reperfusion, we identified a novel mechanism that could contribute to the neuroprotective effect of mild hypothermia. The major findings of this study are that (1) intraischemic mild hypothermia diminishes the induction and accumulation of cell-killing oxidative DNA lesions in the brain after severe focal ischemia and reperfusion; and that (2) mild hypothermia attenuates the activation of both PARP-dependent and p53-dependent pro-death signaling pathways in the ischemic brain. These results suggest that intraischemic mild hypothermia may achieve a neuroprotective effect by preventing DNA damage-mediated ischemic neuronal death.

In the present study, we investigated the effect of intraischemic mild hypothermia on the induction profiles of three different types of oxidative DNA lesions, namely 8-oxodG, AP site, and DNA single-strand breaks (SSB), after focal cerebral ischemia and reperfusion. These DNA lesions result from direct attacks by reactive oxygen or nitrogen species (ROS and RNS) that are overproduced in neurons predominantly during postischemic reperfusion (19) and are sensitive indicators of cellular oxidative injury in the ischemic brain (6, 8). Consistent with our previous results (10, 14), the levels of all three DNA lesions were significantly increased in both cortex and caudate during early reperfusion stages after MCAO, preceding any evidence of cell death in these brain regions. Remarkably, mild hypothermia, applied selectively to the brain during the 2-hr MCAO, completely prevented the induction of 8-oxodG, AP site and SSB in the ischemic cortex and, to a lesser extent, attenuated the induction of lesions in the caudate. This effect of mild hypothermia correlates with its neuroprotection against infarction in the cortex and caudate, respectively.

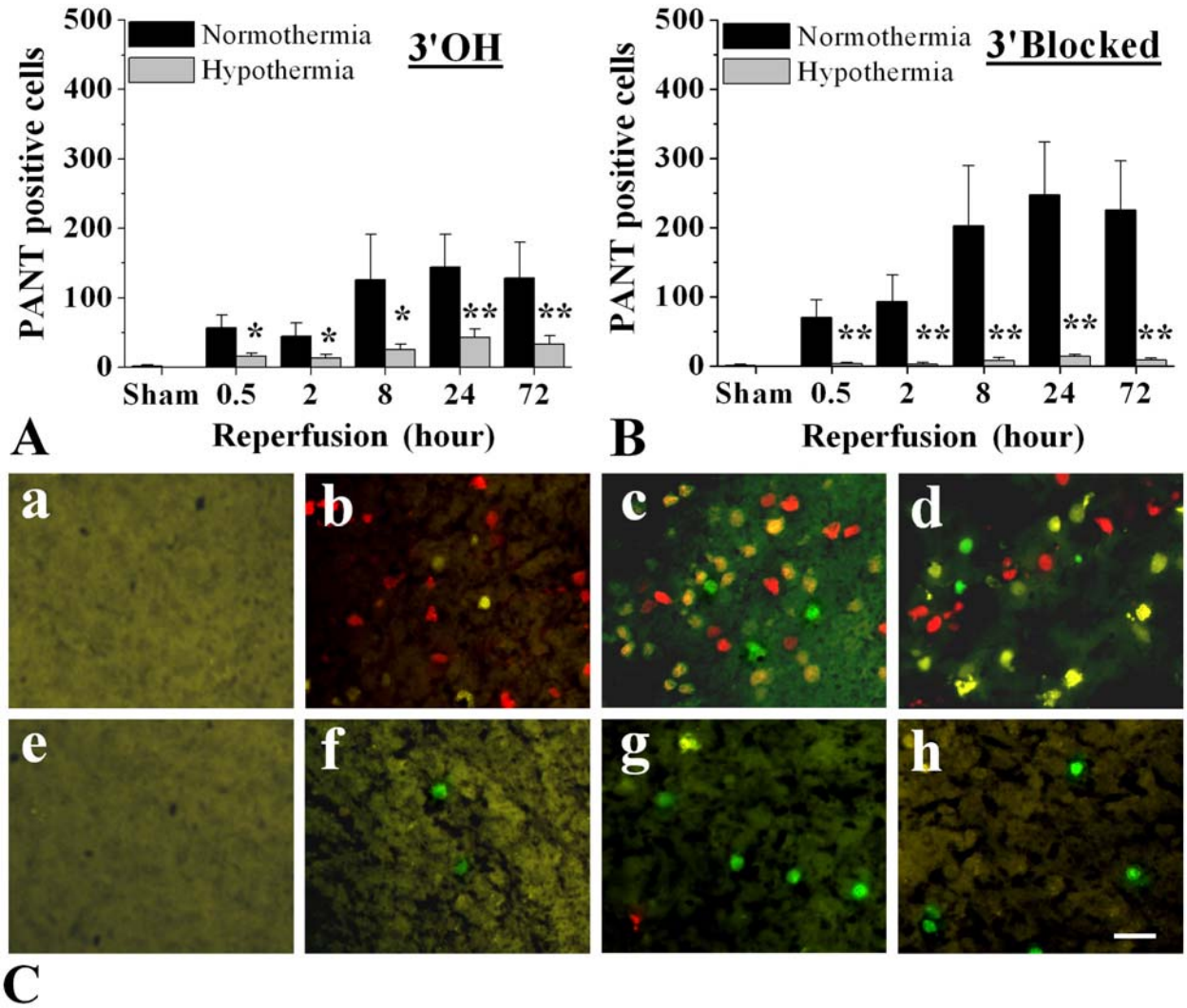

Figure 4. Intraischemic hypothermia attenuates the induction of DNA single-strand breaks in the brain following focal ischemia and reperfusion. A-B, quantitative analysis of the induction of DNA single-strand breaks (SSB) containing either the 3' OH (A) or 3' blocked group (B) in cells in the parietal cortex at 0.5, 2, 8, 24 and 72 hr after 2-hr MCAO in brains. Results are expressed as the amounts of SSB cells per high-power microscopic field (magnification x200). *p < 0.05, **p < 0.01 *versus* normothermia controls (n = 6 per group). C, representative images show the cortical induction of DNA SSB containing either the 3' OH (green fluorescence) or 3' blocked group (red fluorescence, double-labeled cells showing yellow fluorescence) in cells at 2 hr (panels b and f), 8 hr (panels c and g), and 24 hr (panels d and h) after ischemia. The non-ischemic control brains show no SSB-positive cells (panels a and e). Note that the number of SSB-positive cells is markedly reduced in hypothermia-treated brains (panels e, f, g, h) as compared to normothermia brains (panels a, b, c, d). Scale bar = 50 μm.

The precise mechanism by which mild hypothermia diminishes oxidative DNA damage after ischemia and reperfusion is not elucidated in this study. However, attenuation of the release of excitatory amino acids, such as glutamate, and reduced formation of reactive oxygen species by hypothermia may be an important underlying mechanism. Glutamate, through the activation of both NMDA and non-NMDA receptors, is known to gate excessive calcium influx into neurons and trigger various ROS-generating enzymes after cerebral ischemia (19). A direct causative link between glutamate excitotoxicity and oxidative DNA strand damage has been demonstrated previously in cultured cortical neurons (20). Moreover, a number of studies have shown that intraischemic mild hypothermia markedly attenuates glutamate release and/or ROS production after cerebral ischemia (1, 21-26). In a study using the 2-hr MCAO and reperfusion paradigm in rats, we found that, when brain temperature was decreased to 32-33^{o}C during ischemia, glutamate efflux was completely abolished in the cortex, but only partially attenuated in the caudate (Chen et al., unpublished data), which was consistent with the pattern of changes in oxidative DNA lesions characterized in this study. Given the crucial role of glutamate excitotoxicity in triggering oxidative stress in cerebral ischemia, the effect of mild hypothermia on ROS formation in the ischemic brain is likely the consequence of inhibiting glutamate release.

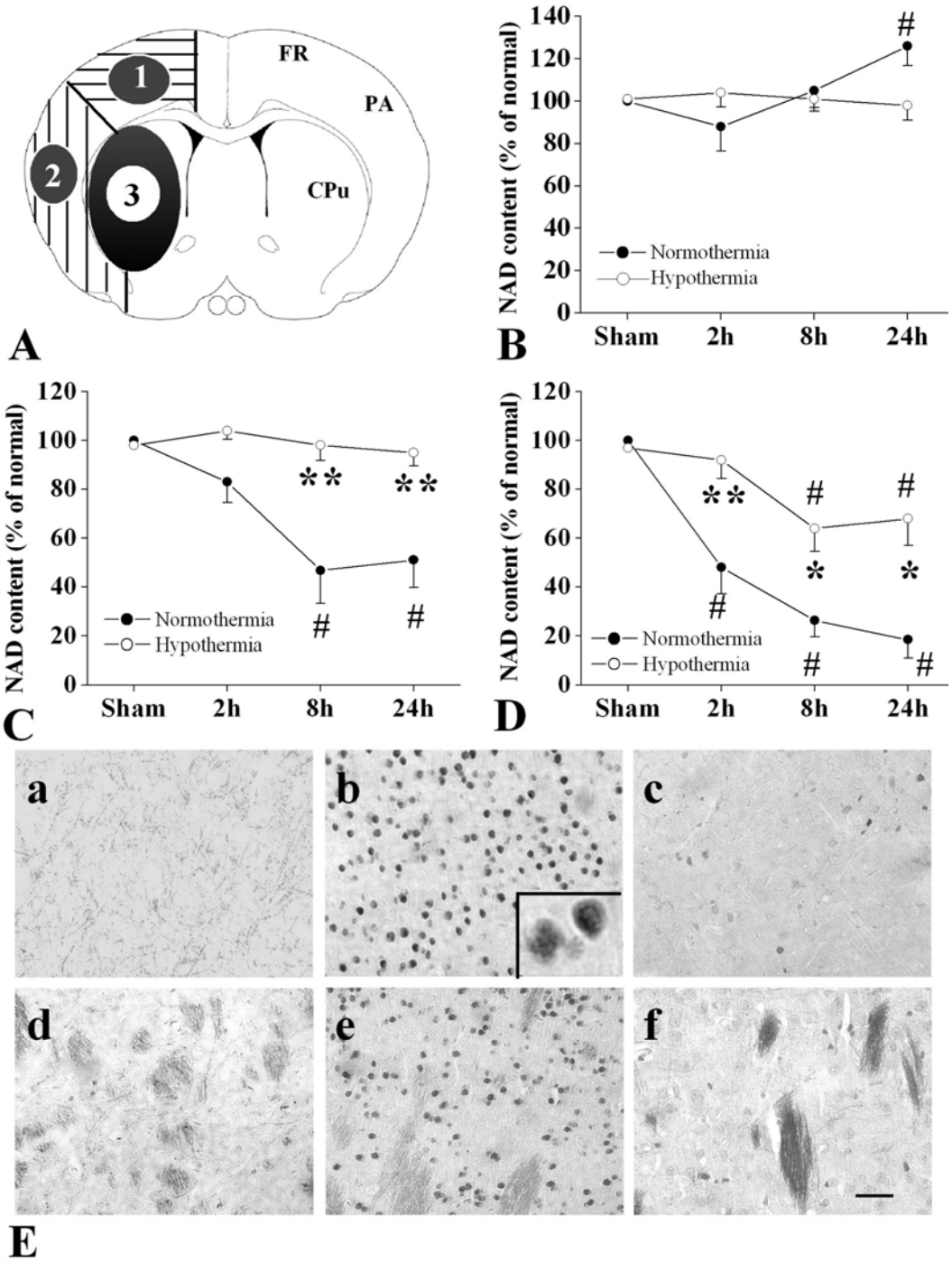

Figure 5. Intraischemic hypothermia attenuates PARP activation in the brain following focal ischemia and reperfusion. A, schematic diagram depicts regions from which tissues were sampled for NAD measurement. Region 1 refers to the cortical ischemic border zone; region 2 refers to the cortical ischemic core; region 3 refers to the caudate-putamen. B-D, changes in tissue NAD contents in region 1 (B), region 2 (C), and region 3 (D) at 2, 8 and 24 hr after 2-hr MCAO, with or without hypothermia treatment (n = 6 per group). *p < 0.05, **p < 0.01 *versus* normothermia group; #p < 0.05 *versus* sham-operated non-ischemic controls. C, representative micrographs show changes in poly(ADP-ribose) immunoreactivity at 8 hr after 2-hr MCAO, with or without hypothermia treatment. Images were taken from region 2 (panels a-c) and region 3 (panels d-f), respectively. Note that poly(ADP-ribose) immunoreactivity is not detectable in non-ischemic cortex (panel a) or caudate (panel d), but it is markedly increased in both cortex (panel b) and caudate (panel e) after normothermic ischemia. The insert in panel b demonstrates the nuclear localization of the immunoreactivity. Ischemia-induced increases in poly(ADP-ribose) immunoreactivity are attenuated in the cortex (panel c) and caudate (panel f) following hypothermia treatment. Scale bar = 50 μm.

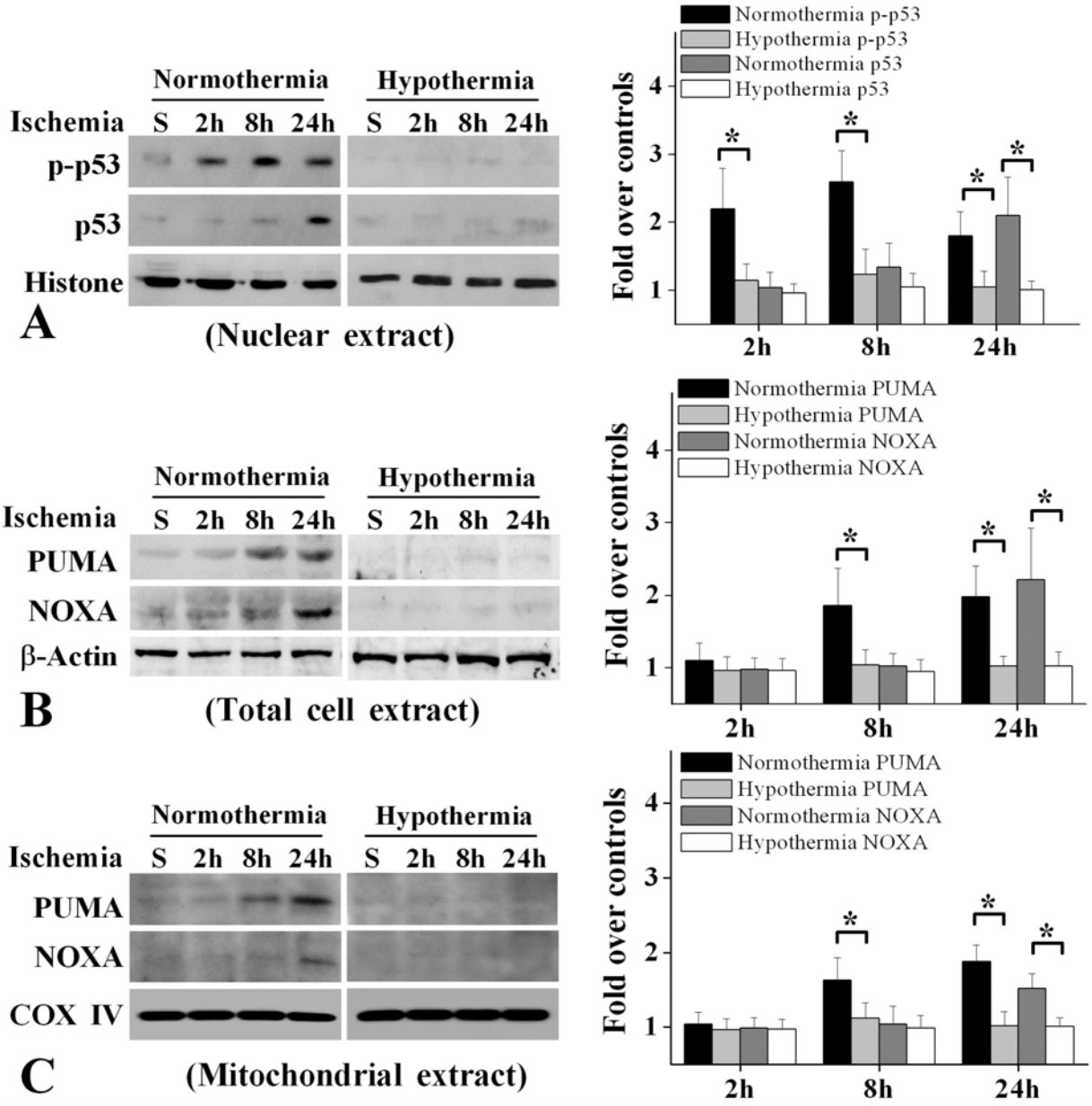

Figure 6. Intraischemic hypothermia attenuates p53-dependent pro-death signaling in the brain following focal ischemia and reperfusion. Western blots were performed using protein extracts prepared from the cortical ischemic border zone (region 1 in Figure 5A) at 2, 8, and 24 hr after MCAO, with or without hypothermia treatment. A, time-dependent increases in the phosphorylation of p53 (Ser46) after ischemia. The levels of total p53 are increased only at 24 hr after ischemia. B, increases in the expression of PUMA and NOXA proteins after ischemia. C, increases in the levels of PUMA and NOXA proteins in the mitochondrial fraction after ischemia. The graphs in the right panel summarize the results from four independent experiments. *$p<0.05$ ($n = 4$ per condition) hypothermia *versus* normothermia. Note that hypothermia abolished the changes in p53, PUMA and NOXA after ischemia.

Emerging evidence has suggested that oxidative damage to genomic DNA plays an important role in neuronal cell death after transient cerebral ischemia (11). Several types of DNA lesions may have remarkable detrimental effects on neuronal cell survival by triggering a cell death cascade. Formation of 8-oxodG is associated with gene mutagenesis, which, when persistently accumulated, may result in partial or complete loss of the functions of the damaged genes (27, 28). AP site, through interrupting gene transcription and DNA synthesis, is directly lethal to cells by inducing p53-dependent apoptosis (29-31). Recently, we found that neurons with decreased levels of AP

endonuclease, the key repair enzyme for AP site, become extremely vulnerable to cell death induced by *in vitro* ischemia (11), indicating that AP site can be a trigger for ischemic cell death. DNA SSB is also a potent blocker of DNA synthesis or gene transcription and, when unrepaired, may potently trigger cell death *via* multiple signaling mechanisms, including PARP-, p53-, and CDK-dependent pathways (17, 18, 32, 33). In agreement with these notions, our current data show that both the PARP-1 and p53 signaling pathways were activated in ischemic brain regions that suffered from persistent DNA damage. Moreover, the data show that mild hypothermia prevented the activation of both pro-death signaling pathways as well as the accumulation of DNA lesions. However, the relative contributions of the PARP-1 and p53 pathways to cell death in the focal ischemic brain may be region-dependent. In the ischemic core, PARP-1 was markedly activated, resulting in the depletion of intracellular NAD^+, which likely leads to necrotic cell death in this region. In contrast, in the ischemic penumbra region (infarct border), the activation of p53 as well as its downstream pro-apoptotic signaling molecules PUMA and NOXA appeared to be the major signaling events contributing to cell death. Therefore, the decreases in oxidative DNA damage by ischemic hypothermia may contribute to the attenuation of both necrosis and apoptosis after focal ischemia and reperfusion.

In summary, the present study demonstrates the potent inhibitory effect of mild hypothermia on the induction of oxidative DNA damage after focal cerebral ischemia and reperfusion. The attenuation of oxidative DNA damage is likely responsible for preventing multiple DNA damage-dependent pro-death signaling pathways under hypothermia. Thus, the attenuation of oxidative DNA damage may be an important mechanism underlying the neuroprotective effect of intraischemic hypothermia against ischemic brain injury.

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Abbreviations: MCAO: middle cerebral artery occlusion; 8-oxodG: 8-hydroxyl-2'-deoxyguanosine; AP site: apurinic/apyrimidinic abasic site; DNA SSB: DNA single-strand break; PARP: poly(ADP-ribose) polymerase; PANT: DNA polymerase I-mediated biotin-dATP nick-translation; PUMA: p53 up-regulated modulator of apoptosis; ROS: reactive oxygen species; RNS: reactive nitrogen species

Key Words: Hypothermia, Neuroprotection, Cerebral ischemia, Reperfusion, Oxidative Stress, DNA damage, Apoptosis, NAD depletion

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