Impaired DNA repair *via* the base-excision repair pathway after focal ischemic brain injury: a protein phosphorylation-dependent mechanism reversed by hypothermic neuroprotection

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TABLE OF CONTENTS

1. ABSTRACT

Cerebral ischemia and reperfusion induces rapid accumulation of oxidative DNA lesions in the brain, which, if not repaired promptly, may trigger cell death. The base-excision repair (BER) pathway is the main mechanism employed by neurons to repair various types of oxidative DNA damage. Recent studies have suggested that the cellular activity of BER is highly regulated (up- or down-regulated) after ischemic brain injury, and this regulation may contribute to the outcome of cell injury. The mechanism through which cellular BER is regulated in response to neuronal injury is currently poorly understood. In the present study, we have examined BER regulation in the rat model of focal ischemic brain injury induced by 2 hr of middle cerebral artery occlusion and 0-72 hr of reperfusion. As determined using cerebral nuclear extracts, focal ischemia resulted in a marked reduction in BER activities, including the overall BER activity, AP endonuclease activity and DNA polymerase-beta activity, indicating functional impairment of the BER pathway. BER reduction occurred as early as 0.5 hr after the onset of reperfusion. Thereafter, BER activity failed to recover, and there were persistent accumulations of apurinic/apyrimidinic abasic sites and DNA single-strand breaks in ischemic tissues. The reduction in BER during the early reperfusion phase (< 6 hr) was not accompanied by any alterations in the levels of essential BER enzymes in brain extracts. However, increased serine- and threonine-specific phosphorylation was detected for both AP endonuclease and DNA polymerase-beta after ischemia, with the time course of serine phosphorylation closely correlated to that of changes in BER activity. Furthermore, dephosphorylation of nuclear extracts with alkaline phosphatase largely restored AP endonuclease and DNA polymerase-beta activities. Taking advantage of the neuroprotective effect of mild hypothermia (33°C), which was induced in the brain during the first 2 hr of reperfusion, we found that the post-ischemic suppression of BER activity is a reversible event. Hypothermic treatment diminished the serine-specific phosphorylation of AP endonuclease and DNA polymerase-beta, promoted BER activities, and attenuated the levels of oxidative DNA lesions after ischemia. These results suggest that the functional impairment of the BER pathway after severe focal cerebral ischemia is due to the loss-of-function post-translational modifications of repair enzymes. Further investigations elucidating the precise mechanism underlying the post-translational regulation of BER enzymes may lead to novel therapeutic strategies for cerebral ischemia.

2. INTRODUCTION

Oxidative DNA damage resulting from direct attacks by reactive oxygen species is a prominent phenomenon occurring during early stages of reperfusion after cerebral ischemia (1-4). Several types of oxidative DNA damage, such as apurinic/apyrimidinic abasic site (AP site) and single-strand breaks (SSB), are highly cytotoxic, and, when not repaired promptly, are capable of triggering cell death through the activation of various signaling pathways (reviewed in (5)). In the brain, oxidative DNA lesions are repaired mainly *via* the base-excision-repair (BER) pathway, which is catalyzed by the sequential actions of a group of DNA repair enzymes, including specific DNA glycosylases, AP endonuclease, DNA polymerase-β, and DNA ligase I or III (6-8). Given that the neuronal genome encounters enormous oxidative damage even under physiological conditions (9, 10), the functional integrity of the BER pathway is essential for neurons to survive. Under oxidative stress, such as that occurring in cerebral ischemia and reperfusion, there are greatly increased demands for BER in preventing the harmful accumulation of oxidative DNA lesions in injured neurons (3, 11).

Recent studies have found that BER in the brain is a highly regulated (up or down) process after ischemia, and its functional status is correlated with the outcomes of brain injury (3, 11). In ischemic penumbra where the tissue is ischemic but devoid of rapid necrosis, BER activities are markedly up-regulated during post-ischemic reperfusion, partially due to the increased expression of BER enzymes (3, 12). Moreover, in the rat model of ischemic preconditioning, in which a tolerance state against ischemic injury is induced by a sublethal ischemia, the tolerant tissue exhibits an increased BER capacity but decreased accumulation of oxidative DNA lesions and attenuation of neuronal cell death (11). In contrast to the BER response in sublethal ischemic injury, the function of BER after lethal ischemic insult appears to be greatly compromised, leading to the speculation that neurons failing to match the demand for increased BER capacity after ischemia would suffer from the accumulation of DNA lesions, impairment of genomic integrity, and, eventually, cell death (3). However, despite the current understanding that alteration in BER activities may have important influence on the outcome of ischemic brain injury, the mechanism underlying the regulation of BER after ischemia is poorly understood.

In this study, we have examined the regulation of BER in the rat model of focal cerebral ischemia and reperfusion. Using an injury paradigm (2 hr of ischemia) that results in infarction in the whole cerebral territory supplied by the middle cerebral artery, we identified a post-translational process responsible for the functional impairment of BER during early stages of post-ischemic reperfusion. Taking advantage of the robust neuroprotective effect of mild hypothermic treatment in the brain, we further found that BER dysfunction after lethal focal ischemia could be largely reversed by preventing the protein modifications of BER enzymes DNA polymerase-β and AP endonuclease.

3. MATERIALS AND METHODS

3.1. Rat model of focal cerebral ischemia

The animal protocols used in this study were approved by the Institutional Animal Care and Use 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 isoflurane-anesthetized male Sprague-Dawley rats (275-310 g), using the intraluminal vascular occlusion of the middle cerebral artery (MCA) as previously described (13). After 2 hr of MCAO, the suture was withdrawn to initiate reperfusion.

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 reperfusion and maintained for 2 hours. Rectal temperature was kept in the range of $36.5\text{-}37.3^{\circ}$C throughout the experiment using a temperature-regulated heating lamp and heating pad.

To verify the neuroprotective effect of mild hypothermia on focal ischemic brain injury, infarct volume was determined at 72 hr after 2-hr MCAO in two groups of rats (n = 6 per group) using the MCID image analysis system as previously described (13). 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.

3.2. Measurement of overall BER activity

The *in vitro* DNA incorporation repair assay was performed to measure the overall BER activity in nuclear protein extracts as described previously (3). This assay examines the ability of nuclear protein extracts to incorporate [^{32}P]dGTP into oxidatively damaged plasmids. The DNA repair substrate used in the assay consisted of purified pcDNA plasmids containing the oxidative adduct 8-oxodG, which were prepared using photoactivated methylene blue (MB) (14). The amounts of 8-oxodG in the plasmids were verified using HPLC-EC, typically in the range of 150-200 8-oxodG/10^5dG.

To perform the repair assay, nuclear protein extracts (quantity of protein as indicated) from cortical tissues obtained at 0, 0.5, 1, 3, 6 or 24 hr after 2 hr of ischemia or 24 hr after sham operation (n = 3 per group) were incubated for 60 min at 32°C in 50 μl of reaction mixture. The reaction mixture consisted of 0.3 μg 8-oxodG-rich pcDNA plasmids, 45 mM HEPES-KOH, 70 mM KCl, 2 mM MgCl$_2$, 1 mM dithiothreitol, 0.4 mM EDTA, 2 mM ATP, 20 μM each dATP, dTTP and dCTP, 8 μM dGTP, 2 μCi of [alpha-^{32}P]GTP (NEN), 40 mM phosphocreatine, 2.5 μg of creatine phosphokinase, 3% glycerol, 20 μg/ml bovine serum

albumin, 2 mM NAD^+, and 1 mM β-mercaptoethanol. The reaction was terminated by the addition of proteinase K (240 μg/ml), 1% SDS and 20 mM EDTA, and the DNA was phenol-extracted from the mixture and dissolved in TE buffer. The samples were treated with 10 units of BamHI (Gibco BRL, Invitrogen, Carlsbad, CA) overnight at 37^oC to linearize the DNA, and then separated by electrophoresis on a 1% agarose gel. Radioactive nucleotide incorporation into the DNA was detected using autoradiography and analyzed by gel densitometry using the MCID image analysis system (St. Catharines, Ontario, Canada). All densitometric values for DNA radiolabels were normalized to values for UV photographs of DNA bands on the same lane.

3.3. Measurement of AP endonuclease activity

The oligonucleotide incision assay was performed to estimate the ability of nuclear protein extracts to remove AP sites. The repair substrate used in this assay was a 50-mer oligonucleotide with a precisely positioned synthetic AP site at position 26 (5'-TCG GTA CCC GGG GAT CCT CTA GAG TOG ACC TGC AGG CAT GCA AGC TTG GC-3'; O = AP site). The oligonucleotide was 5'-end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP, and the reaction mixture was passed through a G-25 spin column to remove the free unlabeled [γ-^{32}P]ATP. The labeled oligonucleotide was then annealed to the complementary oligonucleotides. The reaction mixture for the incision assay contained 40 mM HEPES-KOH (pH 7.6), 75 mM KCl, 2 mM DTT, 1 mM EDTA, 0.1 mg/ml BSA, 2 mM $MgCl_2$, 20 μM zinc acetate, 10% glycerol, 300 fmol of ^{32}P-labeled DNA duplex, and nuclear protein extracts in the indicated amounts. The reaction was incubated at 32^oC for 20 min, and then terminated. The DNA was ethanol-precipitated and then resuspended in formamide dye containing 90% formamide, 0.002% bromphenol blue and 0.002% xylene cyanol. The samples were heated to 80^oC for 2 min and subjected to electrophoresis on a denaturing 20% polyacrylamide gel containing 7 M urea. The incision products were analyzed by autoradiography and densitometry analysis.

3.4. Measurement of DNA polymerase-beta activity

This assay was performed as described previously (3, 11). To perform the assay, the 50-mer oligonucleotide (300 fmol) containing a uracil at position 26 (sequence: 5'-TCG GTA CCC GGG GAT CCT CTA GAG TUG ACC TGC AGG CAT GCA AGC TTG GC-3') was annealed to the complementary oligonucleotide. The DNA duplex was subjected to lesion-digestion using purified UDG (5 U) and endonuclease IV (10 U) at 37^oC for 15 min, and then the mixture was heated to 55^oC for 10 min to inactivate UDG and endonuclease IV. This reaction produced a single-nucleotide nick at position 26 in the DNA duplex, which subsequently served as the repair substrate for DNA polymerase-β. Nuclear extracts at the indicated protein concentrations were then incubated with this repair substrate in the same buffer as above with the additions of 2 μCi of [α-^{32}P]CTP, 40 mM phosphocreatine, 2.5 μg of creatine phosphokinase, 3% glycerol, 2 mM NAD, and 1 mM β-mercaptoethanol. The reaction was carried out at 32^oC for 60 min before it was terminated by adding an equal volume of loading buffer and heating to 80^oC for 2 min. The reaction products were separated in a 15% polyacrylamide gel containing 7M urea, and detected using autoradiography.

3.5. Western blot analysis

Rats were sacrificed at 0.5, 1, 3, 6 and 24 hr after 2 hr of ischemia or 24 hr after sham operation (n = 3 per experimental condition). Cortices were dissected and subjected to nuclear protein extraction and Western blot analysis. The working dilutions for the following antibodies were per the manufacturers' suggestions: DNA polymerase-β monoclonal and DNA ligase I monoclonal (NeoMarkers, Fremont, CA), and APE monoclonal (Novus Biologicals, Littleton, CO).

3.6. 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 (3, 15). 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.

3.7. Detection of DNA single-strand breaks

The DNA polymerase I-mediated biotin-dATP nick-translation (PANT) assay was performed as previously described (1) on fresh-frozen sections from brains subjected to 2 hr of MCA occlusion and 0.5, 1, 3, 6, and 24 hr of reperfusion, with or without hypothermic treatment (n = 6 per time point). In brief, sections were incubated in a moist-air chamber at 37°C for 90 min with the PANT reaction mixture containing 5 mM $MgCl_2$; 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). PANT-positive cells were quantified using a computerized scanning program (MCID, St. Catharines, Ontario, Canada) as described previously (1).

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.
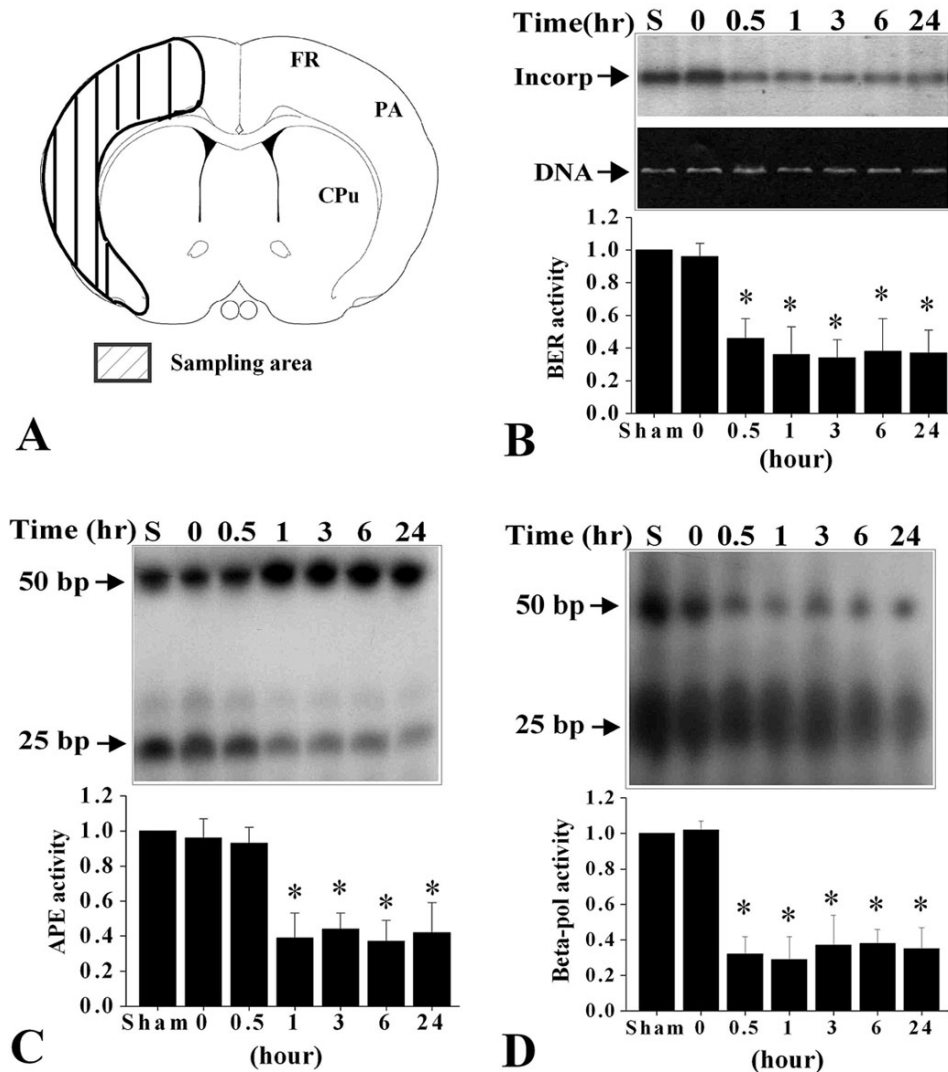
Figure 1. Reduction of BER activities in cerebral cortex after focal ischemia and reperfusion. A, schematic diagram depicts regions from which cortical tissues were sampled for DNA repair assays. FR, frontal cortex; PA, parietal cortex; Cpu, caudate-putamen. B, representative autoradiograph shows ischemia-induced reduction in overall BER activity in cortical nuclear extracts. Samples were obtained after sham operation (S) or after the indicated duration of post-ischemic reperfusion. The ethidium bromide (EB)-stained gel serves as the DNA loading control. C, autoradiograph shows decreased AP endonuclease activity in cortical nuclear extracts after ischemia. In all experiments, equal amounts of DNA substrates (300 fmol) and protein (10 μg) were used in each reaction, and the 25-bp oligo was the specific cleavage product of the assay. D, autoradiograph shows decreased DNA polymerase-β activity in cortical nuclear extracts after ischemia. Equal amounts of protein (10 μg) were used in each reaction; the 50-bp oligo represents the final repair product (DNA synthesis and ligation). The graph below each autoradiograph (panels B-D) illustrates the relative levels of BER activities in the cortex as a function of reperfusion duration, determined by optical density measurements from three independent experiments. All data are mean ± SD. # p < 0.05 *versus* sham non-ischemic controls.

4. RESULTS

4.1. Reduction in BER activities during post-ischemic reperfusion

The first objective of this study was to determine whether the activities of the BER pathway were altered after severe focal cerebral ischemia. Two hours of MCAO produces infarction in both cortex and caudate-putamen within 24 hr, and it has been shown that this injury paradigm induces the accumulations of lethal oxidative DNA lesions, such as AP sites and DNA single-strand breaks, during early and late stages of post-ischemic reperfusion (11). We reasoned that the accumulation of oxidative DNA lesions in this model might be due to both increased DNA damage and impaired DNA repair process. To test this hypothesis, the *in vitro* DNA incorporation repair assay was performed to measure the overall BER activity (which requires the actions of all BER steps) in cortical nuclear extracts (Figure 1A) obtained at 0, 0.5, 1, 3, 6 and 24 hr after 2 hr of MCAO or sham operation. As
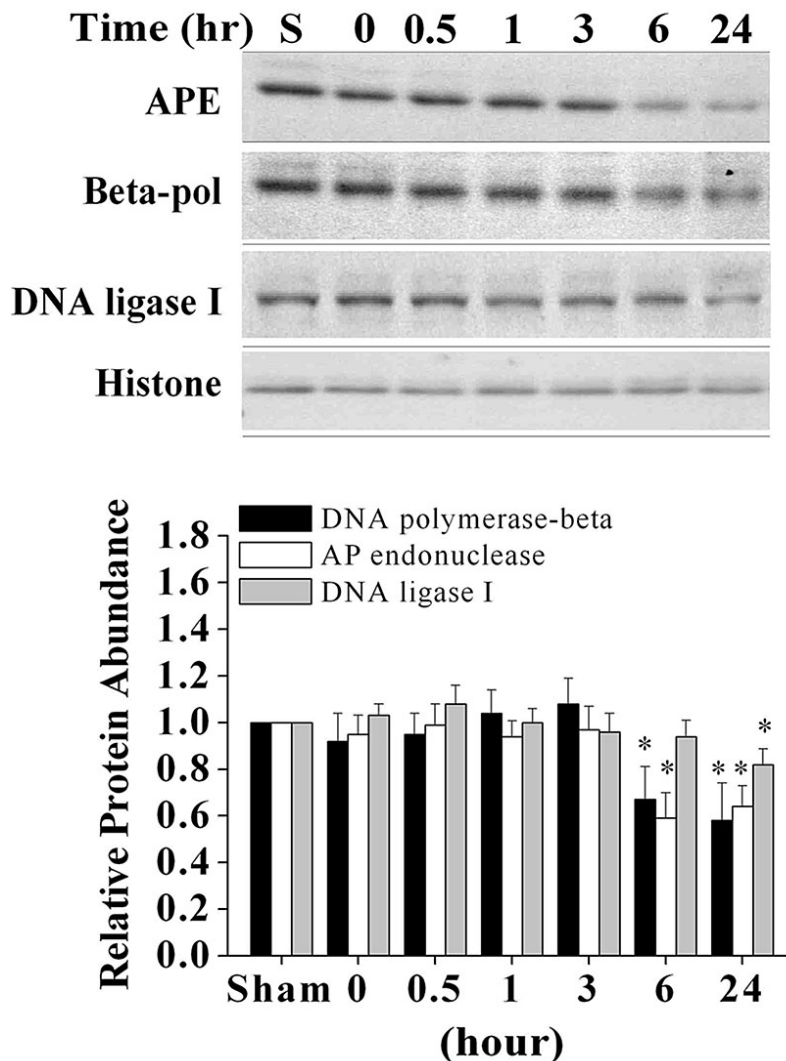
Figure 2. Alteration of the levels of BER enzymes in cerebral cortex after focal ischemia and reperfusion. Representative Western blots show the expression levels of three major BER enzymes in cortical nuclear extracts after ischemia and different durations of reperfusion. Equal amounts of protein (50 μg) were used for each condition. The graph illustrates the relative abundance of BER enzymes analyzed based on optical density measurements in autoradiographs from three independent experiments. All data are mean ± SD. *p < 0.05 *versus* sham controls.

shown (Figure 1B), the DNA repair-synthesis activity was unchanged at the end of MCAO (0 hr of reperfusion), as compared to the sham non-ischemic brains. However, the activity was markedly decreased at 0.5 hr of reperfusion, and this reduction remained un-recovered thereafter, suggesting that the BER pathway was permanently impaired in the ischemic brain.

To determine whether the reduced BER activity after ischemia was due to the dysfunction of AP site cleavage and DNA repair-synthesis, the two key steps in the BER pathway, we measured AP endonuclease and DNA polymerase-β activities in nuclear extracts. Both activities showed marked and persistent reduction after ischemia. The temporal profile of changes in DNA polymerase-β activity after ischemia correlated closely with that of overall BER activity, showing a persistent reduction

that began at 0.5 hr of reperfusion (Figure 1D), whereas a persistent reduction in AP endonuclease activity began to be detectable at 1 hr of reperfusion (Figure 1C).

4.2. Reduction in BER activities independent of altered expression of repair enzymes

Our next objective was to determine whether the reduced BER activity after severe focal ischemia was associated with reduced expression of essential BER enzymes after ischemia. Therefore, Western blot analysis was performed to examine the expression of AP endonuclease, DNA polymerase-β, and DNA ligase I in the brain (Figure 2). The results revealed that the protein levels of AP endonuclease and DNA polymerase-β were unchanged during the first 3 hr of reperfusion, but the levels were significantly decreased at 6 and 24 hr of reperfusion. The expression of DNA ligase I was
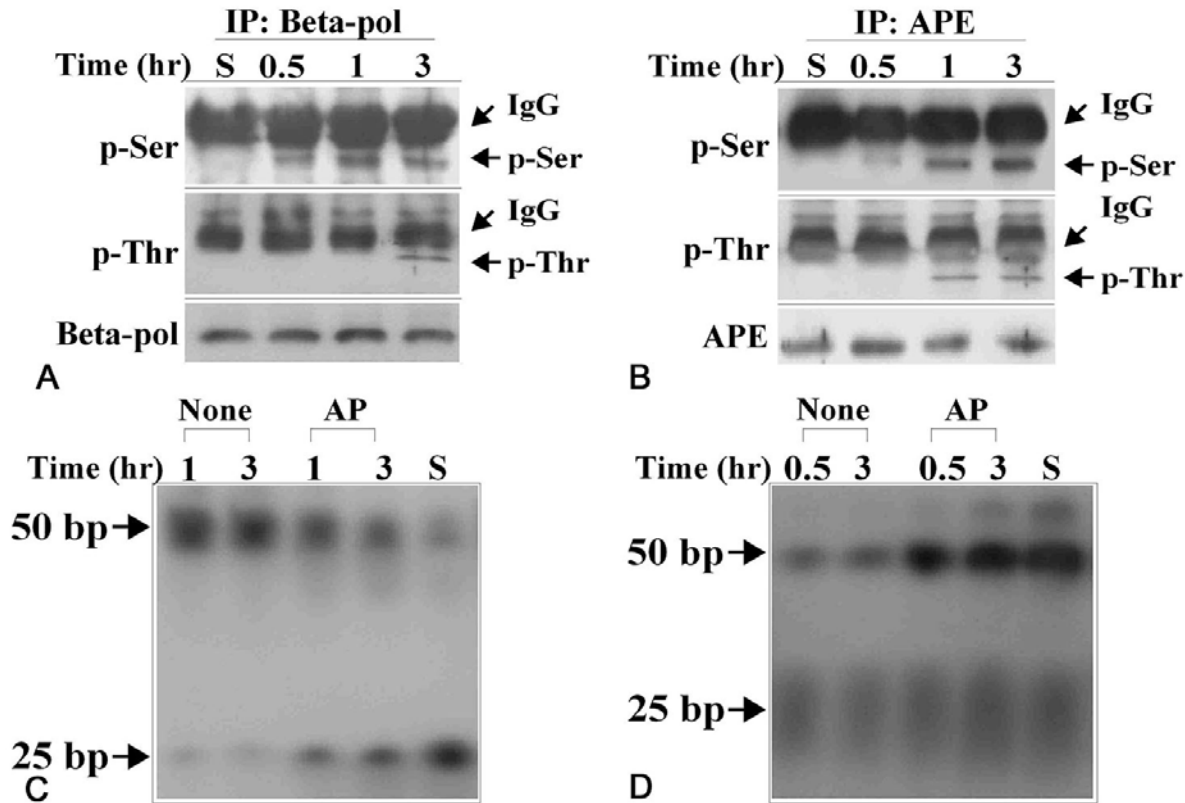
Figure 3. Post-translational alterations of BER enzymes after focal ischemia and reperfusion. A, increased protein phosphorylation of DNA polymerase-β (beta-pol) on serine and threonine residues after ischemia. Nuclear extracts were immunoprecipitated using the anti-beta-pol antibody, and then immunoblotted with antibodies against phosphorylated serine (p-Ser) and phosphorylated threonine (p-Thr), respectively. B, increased protein phosphorylation of AP endonuclease (APE) on serine and threonine residues after ischemia. C, representative autoradiograph shows the partial restoration of APE activity in nuclear extracts (1 or 3 hr after ischemia) treated with alkaline phosphatase (AP), as compared to the sham non-ischemic control. D, representative autoradiograph shows complete restoration of DNA polymerase-β activity in nuclear extracts (0.5 or 3 hr after ischemia) treated with alkaline phosphatase (AP), as compared to the sham non-ischemic control. All autoradiographs are representatives of at least 2-3 independent experiments with similar results.

unchanged until 24 hr after ischemia, where a modest decrease was detected.

4.3. Phosphorylation-mediated inactivation of BER enzymes after ischemia

Based on the results of Western blot analysis, we hypothesized that the reduction of BER activities after ischemia might be due to post-translational inactivation of the repair enzymes. To determine whether protein phosphorylation was involved in the regulation of BER enzymes, immunoprecipitation was performed for AP endonuclease, DNA polymerase-β and DNA ligase I using nuclear proteins obtained at 0.5, 1 and 3 hr after ischemia, and the phosphorylation status of these enzymes was analyzed by immunoblotting against phosphorylated serine (p-Ser), phosphorylated threonine (p-Thr), and phosphorylated tyrosine (p-Tyr). Increased phosphorylation on serine and threonine was detected in ischemic samples for DNA polymerase-β (Figure 3A) and AP endonuclease (Figure 3B), but not for DNA ligase (data not shown). In DNA polymerase-β immunoprecipitates, p-Ser immunoreactivity appeared at 0.5-3 hr after ischemia,

whereas p-Thr immunoreactivity appeared only at 3 hr. In AP endonuclease immunoprecipitates, both p-Ser and p-Thr immunoreactivity appeared at 1 and 3 hr after ischemia.

To determine whether the increased phosphorylation of DNA polymerase-β and AP endonuclease after ischemia was related to the reduction of enzymatic activity, DNA repair assays were performed using nuclear proteins with or without pretreatment with alkaline phosphatase. Alkaline phosphatase treatment completely restored the DNA polymerase-β activity in nuclear extracts to non-ischemic levels (Figure 3D); in contrast, alkaline phosphatase treatment only partially restored the AP endonuclease activity (Figure 3C).

4.4. Post-ischemic hypothermia restores BER activity

To determine whether the functional impairment of BER after ischemia was a reversible or irreversible event, mild hypothermia was induced in the brain at the beginning of reperfusion for up to 2 hr (Figure 4A). This experimental paradigm has shown robust neuroprotective
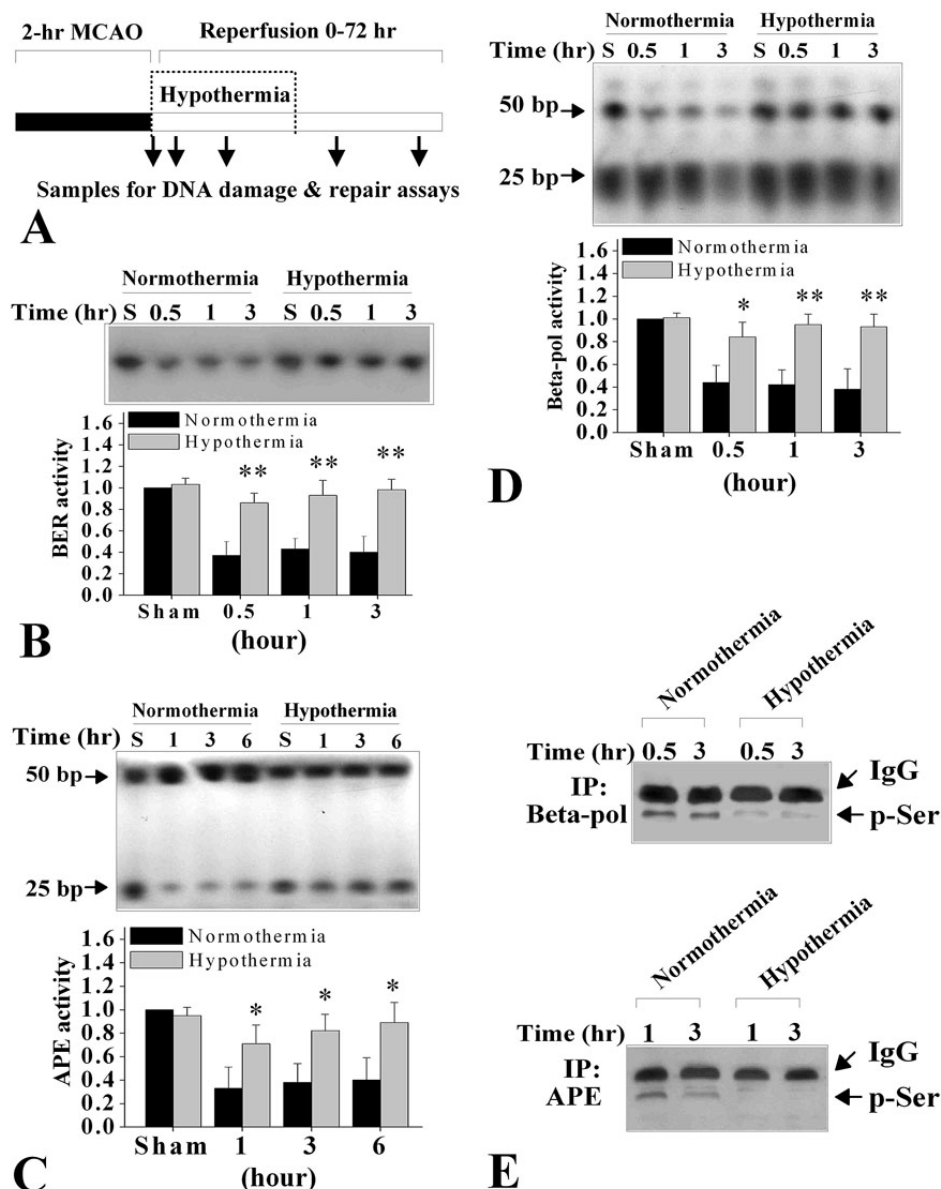
Figure 4. Post-ischemic hypothermia enhances BER activities. A, schematic diagram illustrates the protocol for hypothermic treatment and tissue sampling for DNA damage and repair assays in the present study. In all hypothermia experiments, selective brain cooling (33°C) was performed for up to 2 hr beginning at the onset of reperfusion. B, effect of hypothermia on the overall BER activity in cortical nuclear extracts at 0.5-3 hr of post-ischemic reperfusion. C, effect of hypothermia on APE activity in cortical nuclear extracts at 1-3 hr of post-ischemic reperfusion. D, effect of hypothermia on DNA polymerase-β activity in cortical nuclear extracts at 0.5-3 hr of post-ischemic reperfusion. The graphs in panels B-D illustrate the quantitative results from three independent experiments for each assay. All data are mean ± SD. *$p < 0.05$; **$p < 0.01$ *versus* normothermia groups. E, effect of hypothermia on protein phosphorylation (serine residues) of DNA polymerase-β (beta-pol) and APE after ischemia. Nuclear extracts were immunoprecipitated with either the anti-beta-pol antibody (top panel) or the anti-APE antibody (bottom panel), and then immunoblotted with an antibody against the phosphorylated serine (p-Ser). The data are representative of two independent sets of experiments with similar results.

effect in the rat model of focal cerebral ischemia (23), as also confirmed in this study. Determined using various DNA repair assays, post-ischemic treatment with mild hypothermia significantly enhanced the overall BER activity (0.5-3 hr after ischemia), AP endonuclease activity (1-6 hr after ischemia), and DNA polymerase-β activity (0.5-3 hr after ischemia) during post-ischemic reperfusion over the levels of normothermia control groups (Figure 4B-D). To determine whether hypothermic treatment affected the increased protein phosphorylation of BER enzymes
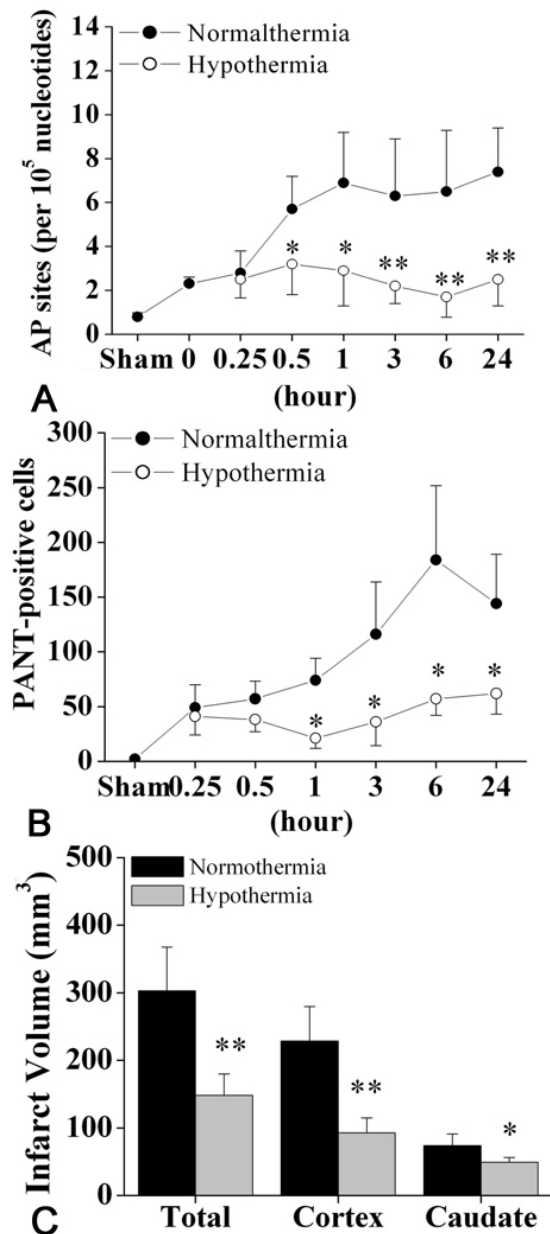
Figure 5. Post-ischemic hypothermia attenuates oxidative DNA damage. A, induction of AP sites in the cortex after 2-hr focal ischemia and reperfusion, with or without hypothermia treatment during the early phase of reperfusion. *p < 0.05; **p < 0.01 *versus* normothermia controls (n = 6 per time point). B, quantitative analysis of the induction of DNA single-strand breaks (SSB) in cells in the frontal/parietal cortex at 0.25, 0.5, 1, 3, 6, and 24 hr after 2-hr MCAO. Results are expressed as the number of SSB cells per high-power microscopic field (magnification x200). *p < 0.05, **p < 0.01 *versus* normothermia controls (n = 6 per group). C, decreased infarct volume (total, cortex, and caudate-putamen) after 2-hr MCAO and 72-hr reperfusion in hypothermia-treated brains. *p < 0.05, **p < 0.01 *versus* normothermia controls (n = 6 per group).

after ischemia, immunoprecipitation was performed using antibodies against DNA polymerase-β (0.5 and 3 hr after ischemia) and AP endonuclease (1 and 3 hr after ischemia), respectively. This was followed by immunoblotting for phosphorylated serine (p-Ser). The results revealed that hypothermic treatment attenuated serine-specific phosphorylation in DNA polymerase-β and AP endonuclease after ischemia (Figure 4F).

4.5. Post-ischemic hypothermia attenuates oxidative DNA damage

Since the results from DNA repair assays suggest that post-ischemic mild hypothermia restored BER activities in ischemic brains, we wanted to determine how the enhanced BER activities affect the levels of oxidative DNA damage after ischemia. Thus, the animals were sacrificed at various time points after 2 hr of MCAO with or without hypothermic treatment (n = 6 per group), and the brain samples were subjected to quantitative measurements for nuclear contents of AP site or PANT staining for the detection of DNA single-strand breaks (SSB). Figure 5A illustrates the temporal profile of AP site induction in cortex during post-ischemic reperfusion. In normothermia rats, the levels of AP site were increased in the brain twofold at the onset of reperfusion, were further markedly increased at 0.5 -1 hr of reperfusion (6-7-fold over non-ischemic controls), and remained elevated thereafter. Hypothermic treatment did not affect the levels of AP site in the first 15 min of reperfusion; however, it significantly inhibited the elevation of AP site levels at all subsequent time points.

Figure 5B shows the effect of hypothermic treatment on the induction of DNA SSB in the brain after ischemia. The temporal profiles of DNA SSB induction in normothermia rats after ischemia were similar to that of AP site. Cells containing DNA SSB were detectable in the ischemic cortex within 15 min of reperfusion, and the number of SSB-positive cells continued to increase thereafter, reaching peak levels at 6 hr after ischemia. Hypothermic treatment significantly decreased the number of SSB-positive cells at 1-24 hr after ischemia.

The effect of mild hypothermia on infarct volume was determined at 72 hr after 2 hr of MCAO (Figure 5C). Post-ischemic hypothermia had a significant neuroprotective effect on ischemic injury, reducing the total infarct volume by approximately 55%. The neuroprotective effect was manifested in both cortex and striatum, reducing infarct volume by ~60% and ~30%, respectively.

5. DISCUSSION

The objective of the present study was to investigate how the BER pathway is functionally regulated in the brain after severe focal cerebral ischemia and reperfusion. Using the rat model of 2-hr middle cerebral artery occlusion (MCAO), we characterized the temporal profile of alterations in BER activities in ischemic cortices at various stages of reperfusion. The major observations are that (1) BER activities are subjected to marked and persistent down-regulation during post-ischemic

reperfusion, indicating the functional impairment of this repair pathway; (2) BER suppression during early stages of reperfusion appears to be due to the effect of increased protein phosphorylation (serine and threonine) on DNA polymerase-β and AP endonuclease; and that (3) mild hypothermia, applied to the brain during post-ischemic reperfusion, restores BER activities after ischemia, and, subsequently, attenuates oxidative DNA damage and infarct sizes after ischemia. These results suggest that post-translational modifications on BER enzymes may contribute to the dysfunction of the BER pathway after severe focal ischemic brain injury, and that this post-ischemic impairment of BER can be reversed by hypothermic neuroprotection.

The data presented here demonstrate that BER activities, including the overall BER activity and the activities of AP endonuclease and DNA polymerase-β, were markedly suppressed in the brain after 2 hr of MCAO and reperfusion. BER suppression began in the early reperfusion stage (0.5 hr) and, subsequently, failed to recover throughout the experiment. This pattern of changes in BER activities was in strong contrast to that after 1 hr of MCAO, where ischemia elicits an up-regulation of cellular BER activities in the frontal/parietal cortex, the main brain region that is affected by MCAO and eventually survives the 1-hr, but not 2-hr, ischemic insult (3). The activation of BER after cerebral ischemia is believed to be an important endogenous adaptive response that protects the brain against the accumulation of lethal oxidative DNA lesions and thus promotes cell survival (3, 11). Similarly, cytoprotective BER activation has also been reported previously in non-neuronal mammalian systems, whereby up-regulation of AP endonuclease or DNA polymerase-β induced by sublethal levels of oxidative stress offers protection against the cytotoxicity of lethal doses of oxidative stress or other DNA-damaging agents (16-18). In contrast, cells deficient in BER activities show increased accumulation of oxidative DNA lesions and markedly decreased cell viability in response to oxidative stress or hypoxic injury (19-21). Accordingly, failure to activate the BER pathway after ischemia could lead to the poor recovery of oxidative lesions and, eventually, to irreversible tissue injury in the ischemic brain. Consistent with this notion, the current study shows that the suppressed BER activities in the lethally injured ischemic cortex were correlated with extremely poor recovery of oxidative lesions and large infarction in this region.

The mechanism underlying the suppression of BER activities after focal cerebral ischemia and reperfusion was investigated in the present study. BER suppression during early reperfusion stages (0.5-3 hr) was not associated with decreased expression of AP endonuclease or DNA polymerase-beta at either mRNA or protein levels. Thus, post-translational modifications of BER enzymes might be involved in the down-regulation of BER activities after ischemia. Using the co-immunoprecipitation approach, we detected increased phosphorylation (serine and threonine) on AP endonuclease and DNA polymerase-β in nuclear extracts from ischemic cortices. The increased phosphorylation of both AP endonuclease and DNA

polymerase-β likely contributed to their inactivation after ischemia, as the recovery of repair activities in nuclear extracts could be achieved by dephosphorylation using alkaline phosphatase. Nevertheless, this study did not investigate the precise phosphorylation sites on AP endonuclease or DNA polymerase-β responsible for the enzymatic inactivation, nor the protein kinase(s) conducting the phosphorylation. A previous study by Tokui *et al.* demonstrated that rat DNA polymerase-β was an excellent substrate for protein kinase C *in vitro* and that the phosphorylation of DNA polymerase-β at two serine residues (44 and 55 from the NH2 terminus) abolished its enzymatic activity (22). It would be tempting in future studies to determine whether protein kinase C is involved in the down-regulation of DNA polymerase-β activity in brain after ischemia. Alternatively, however, activation of another protein kinase or inactivation of a specific phosphatase could be responsible for the observed protein phosphorylation of DNA polymerase-β or AP endonuclease. Although the increased serine phosphorylation on AP endonuclease and DNA polymerase-β showed a similar temporal profile after ischemia, the difference in the time course of threonine phosphorylation suggests the possibility that different protein kinases might be involved in the regulation of these two repair enzymes.

We have examined the effect of hypothermic treatment on ischemia-induced suppression of BER activities and the induction of oxidative DNA damage in the brain. Brain cooling to 33°C was achieved within 15 min after the onset of post-ischemic reperfusion; this delayed intervention paradigm was employed to selectively test its influence on reperfusion-specific events in the brain. The results showed that the loss of DNA polymerase-β and AP endonuclease activity during the early phase of reperfusion could be reversed by hypothermia completely and partially, respectively. The data also showed that hypothermic treatment attenuated the serine-specific phosphorylation of both repair enzymes in the ischemic brain, thus supporting the role of protein phosphorylation in the negative regulation of DNA polymerase-β and AP endonuclease activities. The mechanism by which hypothermia conferred its dephosphorylation effect on DNA polymerase-β and AP endonuclease is unknown; but this effect could be attributable, at least in part, to its ability to reduce the production of reactive oxygen species (ROS) after ischemia (23-26). It is well established that, under oxidative stress, ROS promote serine/threonine phosphorylation *via* the activation of specific protein kinases or inactivation of protein phosphatases in neurons. Taken together, these results suggest that, through inhibiting ROS production, hypothermic treatment attenuates the accumulation of harmful oxidative DNA lesions after ischemia by both decreasing the induction of DNA damage and promoting the repair process.

In summary, the current study identified serine/threonine-specific phosphorylation of DNA polymerase-β and AP endonuclease as the potential mechanism underlying the functional impairment of BER in the brain after focal cerebral ischemia and reperfusion.

BER dysfunction may contribute directly to the accumulation of cell-killing oxidative DNA lesions in the ischemic brain. However, BER dysfunction during the early reperfusion phase appears to be a reversible process that is preventable by hypothermic neuroprotection. Further investigation elucidating the precise mechanism by which BER enzymes are regulated after ischemia may help in the discovery of novel therapeutic targets for ischemic brain injury aiming to reduce the harmful effect of oxidative DNA damage.

6. ACKNOWLEDGMENTS

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Abbreviations: MCAO: middle cerebral artery occlusion; AP site: apurinic/apyrimidinic abasic site; DNA SSB: DNA single-strand break; PANT: DNA polymerase I-mediated biotin-dATP nick-translation; ROS: reactive oxygen species; BER: base-excision repair

Key Words: Hypothermia, Neuroprotection, Cerebral Ischemia, Reperfusion, Oxidative Stress, DNA damage, DNA Repair, Base-Excision Repair, AP Endonuclease, DNA polymerase-β

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