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Oxidative DNA damage precedes DNA fragmentation after experimental stroke in rat brain

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Abstract

Experimental stroke using a focal cerebral ischemia and reperfusion (FCIR) model was induced in male Long-Evans rats by a bilateral occlusion of both common carotid arteries and the right middle cerebral artery for 30–90 min, followed by various periods of reperfusion. Oxidative DNA lesions in the ipsilateral cortex were demonstrated using *Escherichia coli* formamidopyrimidine DNA N-glycosylase (Fpg protein)-sensitive sites (FPGSS), as labeled *in situ* using digoxigenin-dUTP and detected using antibodies against digoxigenin. Because Fpg protein removes 8-hydroxy-2'-deoxyguanine (oh8dG) and other lesions in DNA, FPGSS measure oxidative DNA damage. The number of FPGSS-positive cells in the cortex from the sham-operated control group was 3 ± 3 (mean $\pm$ SD per mm²). In animals that received 90 min occlusion and 15 min of reperfusion (FCIR 90/15), FPGSS-positive cells were significantly increased by 200-fold. Oxidative DNA damage was confirmed by using monoclonal antibodies against 8-hydroxy-guanosine (oh8G) and oh8dG. A pretreatment of RNase A (100 μ g/ml) to the tissue reduced, but did not abolish, the oh8dG signal. The number of animals with positive FPGSS or oh8dG was significantly ($P < 0.01$) higher in the FCIR group than in the sham-operated control group. We detected few FPGSS or oh8dG-positive cells in the animals treated with FCIR of 90/60. No terminal UTP nicked-end labeling (TUNEL)-positive cells, as a detection of cell death, were detected at this early reperfusion time. Our data suggest that early oxidative DNA lesions elicited by experimental stroke could be repaired. Therefore, the oxidative DNA lesions observed in the nuclear and mitochondrial DNA of the brain are different from the DNA fragmentation detected using TUNEL.

Keywords

hydroxyl radicals; oxidative DNA damage; neurotoxicity; reactive oxygen species; stroke

EXPERIMENTAL STROKE MODELS have implicated several initiators in the cascade of events that lead to functional or structural brain damage (1). These initiators include decreased levels of intracellular ATP, low pH, and increased levels of extracellular glutamate, intracellular calcium ions, proteases, and/or free radicals. Free radicals are known to damage proteins, lipids, and nucleic acids. The conventional measurement of free radicals has shown that free radicals are produced within 30 min of focal ischemia (2) and that they are mostly produced in the penumbral region (3). Nitric oxide, a free radical of oxygen, appears to increase during focal

ischemia (4-6). Reactive nitric oxide may combine with superoxide ion to form peroxynitrite, which generates 3-nitrotyrosine in protein. Measurements of 3-nitrotyrosine show an equal distribution of peroxynitrite within the ischemic area (7). Peroxynitrite is also known to cause oxidative damage nucleic acids (oh8G, oh8dG, and DNA strand breaks) either directly or indirectly (8-11).

Two mechanisms have been proposed to account for the damage to nucleic acids observed after cerebral ischemia and reperfusion. The first is mediated by nonspecific nucleases (12-14). The damage, which is often referred to as DNA fragmentation resulting in cell death, is found in the nuclear DNA and is not reversible (15). DNA fragmentation can be activated by proteases (12,13) or by neuronal nitric oxide synthase (11,16-19) and becomes apparent at least a few hours to a few days after cerebral ischemia, depending on the duration of ischemia. The second type is oxidative DNA damage that occurs early after ischemia (within the first 30 min of reperfusion) (9-11). In addition to DNA strand breaks (11,15), this type of DNA damage consists of base modifications (9,10) and DNA lacking a base (11). Evidence suggests that reactive oxygen species, most likely nitric oxide, superoxide ions, and hydroxyl radicals (8), mediate this second type of nucleic acid damage, which is often referred to as oxidative DNA damage (8-11,20). These DNA lesions are similar to those found after ionizing radiation (8) and are generally reversible by DNA repair mechanisms (21), with the exception of those in RNA (10,22). On the other hand, oxidative DNA lesions in the mitochondrial DNA (mtDNA) of the human brain accumulate with age (23). It is not clear how the brain repairs oxidative DNA lesions in both the mitochondria and nuclei, although evidence suggests DNA repair processes exist in general population (21,24-27).

We have detected oh8G (and its deoxy form, oh8dG), 8-hydroxyadenine, 5-hydroxycytosine, and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) in purified DNA from C57BL6 mouse brains that have been subjected to transient forebrain ischemia (9,10). One of these lesions, oh8G/oh8dG, is widely accepted as an indicator of DNA damage generated by oxidative stress (28). Although antibodies to oh8G/oh8dG are available for immunohistochemistry, these antibodies react strongly to lesions in RNA and DNA (10,29, 30). An assay to detect DNA lesions that are sensitive to specific repair enzyme *in vitro* has been used by many laboratories (20,26-28), including ours (9-11). This assay, however, did not provide the location of cell injury. In this article, we describe a novel assay that could detect oxidative DNA lesions based on their sensitivity to Fpg protein *in situ* and bypassed extensive DNA purification. Because oh8dG and FapyGua are substrates for *E. coli* Fpg protein (31), we used this assay to detect Fpg protein-sensitive sites (FPGSS) after experimental stroke in a focal cerebral ischemia and reperfusion (FCIR) model.

MATERIALS AND METHODS

Stroke model

Anesthesia was induced with pentobarbital sodium (Nembutal, 80 mg/kg i.p.) 10 min before surgery. The right middle cerebral artery and both common carotid arteries (the focal cerebral ischemia model) of male Long-Evans rats (Harlan, Indianapolis, Minn., weighing 200–225 g) were occluded for 30–90 min to simulate stroke (32,33). The occlusion was then released to allow reperfusion of the affected area. Housing and anesthesia concurred with guidelines established by the institutional animal welfare committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals, USDA Regulations, and with the American Veterinary Medical Association Panel on Euthanasia guidelines. Body temperature was monitored and maintained at $37 \pm 0.5^\circ\text{C}$; postoperative animal care was as described previously (34,35). All animals were kept in well-ventilated incubators at $24 \pm 0.5^\circ\text{C}$ during the reperfusion period.

Animals, under general anesthesia, were killed as described previously (10,32-35) at the end of the reperfusion period. For immunohistochemical analyses, the animals were perfused with 200 ml each of saline followed by two fixatives in sequence (fixative A: 0.8 g NaOH, 8 g paraformaldehyde, and 1.64 g sodium acetate in 200 ml distilled H₂O, adjusted with 50% glacial acetic acid to pH = 6.5; fixative B: 1.4 g NaOH, 14 g paraformaldehyde, and 13.35 g borax, adjusted to pH = 9.5 with 50% HCl) and cryoprotected in 20% sucrose in fixative B overnight at 4°C as described previously (10,34,35). Brain slices were prepared within 24 h and stored at -20°C for no longer than 4 wk before analysis.

Detection of oxidative DNA lesions *in situ*

Oxidative DNA lesions caused by peroxynitrite or hydroxyl radicals include base modifications and DNA strand breaks (single-strand breaks [SSB] and three types of double-strand breaks [DSB] including those with protruding 5' ends [5'DBS], protruding 3' ends and with blunt ends). *In vitro*, some base modifications such as oh8G/oh8dG are known to be the substrate of *E. coli* Fpg protein (31), which in turn generates SSB. The 3'-PO₄ termini of SSB generated by Fpg protein can be treated with phosphomonoesterase or exonuclease III to remove the 3' PO₄ ends and creating 3'OH ends (36), then labeled using DNA polymerase-I with dNTP plus digoxigenin-dUTP (dig-dUTP). The specificity of this assay depends on the fact that DNA polymerase-I, which is template dependent, incorporates dNTP (with dig-dUTP that replaces TTP) on the 3'OH termini of the SSB or 5'DSB but not on the other two forms of DSB. The presence of dig-dUTP on the newly synthesized strand is then detected using FITC conjugates of the antibody against digoxigenin. Because this assay locates those sites that have been excised by *E. coli* Fpg protein, we refer to these sites of oxidative injury as Fpg protein-sensitive sites (FPGSS) (9,10,21).

Fpg protein was purified as described previously (9,10) and contained no detectable amount of nonspecific nucleases. Coronal sections of 28 animal brains were obtained 1.8–4.8 mm posterior to the bregma. At least four 15-micron tissue sections from each animal were assayed for FPGSS in each experiment. The tissue sections were treated with proteinase K (0.02 mg/ml for 30 min at 37°C). After they were washed extensively, the samples were dehydrated using 70, 95, and 100% ethanol in series (each for 3 min at room temperature), followed by vacuum drying. Before adding Fpg protein, the samples were rehydrated in the incubation buffer (10 mM Tris HCl, pH 7.4, 50 mM KCl). The tissue sections were divided into two groups: one received Fpg protein (1 µg in each of three 20-min intervals, or a total of 3 µg in 1 h incubation) in the incubation buffer (10 mM Tris HCl, pH 7.4, 50 mM KCl), and the other received buffer only. In each detection, animals that underwent the same surgical treatment but received no FCI were used as controls. In addition, a sample from each animal was incubated in buffer to detect pre-existing 3'OH termini in SSB and 5'DSB. Both groups were incubated at 37°C for 1 h. After extensive washes in 10 mM Tris HCl (pH 7.4), the tissue sections were treated with endonuclease-free Kornberg DNA polymerase I (10 U per section, Boehringer Mannheim, Indianapolis, Ind.) with dNTP minus dTTP (each at 200 µM) plus dig-dUTP (20 µM), DTT (1 mM), 50 mM Tris HCl (pH 7.8), and 2 mM MgCl₂ for 1 h at 37°C.

Preparation of hybridoma line 8G-14

To confirm that the ipsilateral FCI cortex contained oxidative DNA lesions, we developed hybridoma that generated the antibody against oh8G/oh8dG lesions. Molecular biology-grade chemicals, including periodate oxidized adenosine, guanosine, cytosine, and uracil, were obtained from Sigma (St. Louis, Mo.). The antigen oh8G (oh8dG or oh8-adenosine) was prepared as described previously (37,38). A conjugate of oh8G and bovine serum albumin (BSA) was prepared in the presence of sodium acetate at pH 7.9 after treatment with periodate at pH 4.6 (39,40). The immunoglobulin M (IgM) antibody was obtained by immunizing mice with periodate-oxidized oh8G/oh8dG coupled to keyhole limpet hemocyanin. The hybridoma

cell lines were prepared as described previously (28,40). Cells were initially screened for positive reactivity with the oh8G-BSA conjugate and for negative reactivity with both BSA and guanosine-BSA conjugates. The antibody-producing hybridoma (8G-14) was one such clone. The conjugate of oh8G-BSA or BSA was tested for its ability to bind the antibody against oh8G/oh8dG using radioimmunoassay. A 96-well plate was coated for 16 h with 50 μ l each of different nucleoside-BSA conjugates, initially at a concentration of 50 μ g protein/ml, and serially diluted 1:2. The BSA conjugates were prepared and diluted in a buffer composed of 50 mM NaPO₄ and 5 mM MgCl₂. The plates were blocked with 5% BSA in PBS. The antibody-containing culture supernatant was incubated on the plates overnight, followed by extensive washing with PBS containing 0.1% BSA. The plates were then incubated with 1:500 diluted rabbit anti-mouse whole Ig (ICN, Costa Mesa, Calif.) for 1 h. The plates were again extensively washed with PBS containing 0.1% BSA and incubated with ¹²⁵I-protein A (110,000 cpm per well) for 1 h. The amount of ¹²⁵I in each well was determined by the addition of 200 μ l of 1 N NaOH to each well and incubating for 1 h at 37°C. At the end of this period, an aliquot of 100 μ l was counted in a gamma counter. The antibody from the conditioned media was tested for its ability to bind specifically with oh8G-BSA and not with BSA blank or BSA conjugates of guanosine, oh8-adenosine, adenosine, cytosine, or uracil. The antibody was later determined to be an IgM antibody.

Determination of oh8G/oh8dG using immunohistochemical methods

The presence of oh8G/oh8dG lesions in the prepared brain tissue slices was determined as described previously using the free-floating method (10). The primary antibody was murine IgM from hybridoma 8G-14 (8G-14 IgM). The conditioned medium containing 8G-14 IgM was concentrated 20-fold using the centricon-50 and diluted 1:10 before use. The secondary antibody was a goat anti-mouse IgM-FITC conjugate (Sigma). Photographs of the fluorescent image were obtained as described previously (9-11). Two independent experiments were performed. Brain specimens that showed a higher fluorescent signal in the ipsilateral (FCI) cortex than in the contralateral (non-FCI) cortex, and in which the fluorescent signal could be abolished or significantly reduced by preadsorption of 8G-14 IgM with oh8G-BSA, were defined as oh8G/oh8dG-positive. The experiments were repeated and confirmed using another antibody against oh8G/oh8dG (QED Bioscience, Inc., Wellesley, Mass.) (8,28,38).

DNA fragmentation

To determine whether FPGSS could be detected as DNA fragmentation, we used terminal UTP nicked-end labeling (TUNEL) staining to detect all strand breaks, including the 3'OH termini of SSB and all forms of DSB. TUNEL assay was used to detect DNA damage in animals with 30 min, 60 min, or 24 h of reperfusion after 90 min of FCI. Because 90 min of FCI produces necrosis within 24 h of reperfusion (32,33), we also examined DNA fragmentation in animals with 1-4 days of reperfusion after 30 min of FCI to avoid interference from necrotic DNA fragmentation (1,41). The incorporation of dig-dUTP to DNA was detected using antibody against digoxigenin-FITC (Oncor, Gaithersburg, Md.). The fluorescent images were digitally captured using a Cooled Color Digital Camera (the SPOT camera, Diagnostic, Sterling Heights, Mich.) and statistically analyzed (10,11).

Statistical analysis

All experiments described in this article were repeated at least twice in tissue samples collected from a minimum of three animals at each time point. Briefly, the fluorescent images were captured on Kodak film (9,35), color prints were made, and the printed images were scanned using a flatbed scanner (HP Picture Scan) into the computer in digitized forms for analysis. In some experiments, the fluorescent images were captured using a Cooled Color Digital Camera. The intensity of green fluorescent signal was analyzed using Adobe PhotoShop 5.0 (10,11).

For FPGSS analysis, cells with at least a threefold increase in the signal value over the background (30 ± 18) in the contralateral cortex using PhotoShop were defined as FPGSS-positive cells. The mean density ($\pm$ sd) of positive cells in the right ipsilateral cortex of each animal (four brain sections per animal, each separated by 50μ) was obtained. Animals with a density of ≥ 150 FPGSS-positive cells per mm^2 (excluding brain surface epithelial cells) were defined as FPGSS-positive animals. If any animals were FPGSS-positive without Fpg protein pretreatment, the animals were defined as positive in non-FPGSS (SSB/5'DSB) and would not be analyzed further.

For oh8G/oh8dG immunoreactivity, we defined animals as oh8G/oh8dG-positive if their ipsilateral cortices showed at least twofold or higher increase in fluorescent signal compared with the contralateral cortex and if the fluorescent signal was abolished or significantly reduced in the negative controls in the same FCIR tissue treated with 8G-14 IgM antibody preadsorbed with antigen oh8G-BSA, 8G-14 IgM antibody deletion, and secondary anti-IgM-FITC deletion.

Cell densities of FPGSS-positive (or oh8dG-positive) were compared in non-FCI and FCIR (90/15 only) animals using Student's *t* test. In addition to Student's *t* test, we also adapted Fisher's exact test, an established method for genetic testing (42). We graded animals as positive or negative for FPGSS and/or oh8dG-immunoreactivity rather than using infarct volume (32,33). The use of infarct volume would have introduced variables for the following reasons. 1) Oxidative DNA damage undergoes continual repair, and individual cells and animals may have different rates of repair that could introduce variability if cellular counts were used at the time when repair of DNA damage is detected. 2) The FCIR model induces edema in the affected cortex, which may alter infarct volume independently of DNA damage, and we could not obtain the correction factor for edema under microscope using a 100x objective. 3) Because there was a gradation of ischemia from the core of the affected cortex to its outermost boundary, known as anoxic depolarization in the core and intra-ischemic depolarizations in the penumbral regions and extrapenumbral cortical regions (1,7). Because of the small number of expected frequencies, we used Fisher's exact test to compare the number of FPGSS-, oh8G/oh8dG-, or TUNEL-positive animals in the control group with the FCIR groups. The level for a statistical significance for all tests was set at $P < 0.05$.

RESULTS

DNA damage and strand breaks *in situ* after FCI

Figure 1 shows that Fpg protein specifically excised oh8dG in DNA oligomer (lanes 3, 4). Moreover, the fragments of oligomer with oh8dG after Fpg protein digestion could not be extended by Klenow enzyme of *E. coli* DNA polymerase-I (lanes 7, 8) but could be extended by two different preparations of endonuclease-free Kornberg DNA polymerase-I (lanes 5, 6, 9, 10). The fact that Kornberg DNA polymerase-I can extend the fragment generated by Fpg protein indicates that the preparation of Kornberg DNA polymerase-I contained phosphomonoesterase and could be used to detect FPGSS in the brain tissue.

When brain sections from animals treated with 90 min of FCI were tested for FPGSS by incubating with Fpg protein, followed by a treatment with Kornberg DNA polymerase-I and labeling substrates, we observed no incorporation of dig-dUTP in the contralateral left (non-FCI) cortex (Fig. 2A), as compared with the ipsilateral right (FCI) cortex (Fig. 2B). Fpg protein or Kornberg DNA polymerase-I alone did not increase the dig-dUTP signal in the brain sections of the control animals (Fig. 3A or B, respectively). Only in the non-FCI brain tissue that was treated with DNase I (to make SSB bearing 3'-OH ends) and with Kornberg DNA polymerase-I did we observe the dig-dUTP signal in the nucleus (Fig. 3C). This result confirmed that the

signal was from dig-dUTP on DNA and that the procedures themselves did not create excessive strand breaks.

Figure 4 shows an increase in the presence of cellular FPGSS in Fpg protein-treated cortices from animals treated with FCIR (four representative cortices from the 33 animals listed in Table 1). We did not observe significant dig-dUTP incorporation in the nuclei of the cortex from sham-operated animals (Fig. 4A [no Fpg protein] and Fig. 4E [with Fpg protein]). The presence of cytosolic green signals without Fpg protein appeared similar to the background shown in Fig. 3A, B and treatment with Fpg Protein did not increase the signal (Fig. 4E). In animals with FCIR, we observed no significant incorporation of dig-dUTP without Fpg protein pretreatment (Fig. 4B, C), but we observed a significant elevation of cellular FPGSS during the first 15 min of reperfusion in Fpg protein-treated tissue (Fig. 4F), and the signal decreased within the next 45 min (Fig. 4H).

The signal of FPGSS in the FCIR animals was located mostly in the nucleus at 90/15 and 90/30 FCIR groups and in the perinuclear (asterisks) regions at 90/60 FCIR group. We also noticed a trace of dig-dUTP incorporation in tissue section without Fpg protein at 30 and 60 min of reperfusion (arrows, Fig. 4C, D), suggesting the presence of non-FPGSS signals at these time periods. The mean density of FPGSS-positive cells (per mm²) in the non-FCI cortex was 3 ± 3 (mean $\pm$ sd) compared with 757 ± 58 in animals with FCIR of 90/15 ($P \leq 0.001$, *t* test). The density of FPGSS-positive cells in FCIR of 90/30 and 90/60 was not calculated because of the presence of non-FPGSS signal (SSB/5'DSB). A total of 7 of 9 non-FCI control animals and 1 of 28 FCI animals were classified as FPGSS-negative animals (Table 1). The remaining 27 FCI animals were either FPGSS-positive ($n=20$) or positive with non-FPGSS signals (SSB/5'DSB, $n=7$) signals. Three of four FCI animals with 120 min of reperfusion contained positive non-FPGSS signals.

Detection of oxidative stress by the presence of oh8G/oh8dG after FCI

To confirm that the presence of FPGSS did in fact indicate oxidative DNA damage in the FCIR cortices, we measured oh8G/oh8dG immunoreactivity in the right FCIR cortex. Two types of antibodies were used: the newly developed 8G-14 IgM and a commercial IgG (10,28,38). Figure 5 shows the oh8G/oh8dG immunoreactivity (green fluorescent signal) in the ipsilateral cortex after FCIR (90/15, $n=3$). No green signal was observed in the right cortex of the control animal (data not shown). The ipsilateral cortices from all of FCI animals (within 30 min of reperfusion after 30, 60, or 90 min FCI) exhibited elevated oh8G/oh8dG immunoreactivity (Table 2). We observed an elevation in oh8G/oh8dG immunoreactivity in the right (ipsilateral) cortex (Fig. 6A), area II of the parietal cortex (Fig. 6B), caudate putamen, cingulum, and corpus callosum (Fig. 6C) of FCIR animals as compared with the non-FCI cortex (Fig. 6D). The green immunoreactivity of oh8G/oh8dG antibody complex was mostly seen in the cytoplasm with trace amounts of staining in the nuclei (arrows, Fig. 6A). The three negative FCIR control animals, which included 1) preadsorption of the IgM antibody with the antigen of the oh8G-BSA conjugate (Fig. 6E), 2) no 8G-14 IgM antibody (Fig. 6F), and 3) no secondary antibody (not shown), reduced or abolished the green fluorescent signal. Indeed, the 8G-14 IgM antibody bound only to the oh8G-BSA conjugate (Fig. 7), indicating the specificity of the IgM antibody. The weak fluorescent signal seen in the nonischemic animals (arrow, Fig. 6D) suggests that a small amount of oh8G lesions existed during normal metabolism (20,30).

Because the IgM antibody stained both oh8G in RNA and oh8dG in DNA, cellular outlines were difficult to discern, making differentiation between the neurons and astrocytes based on morphology generally not possible under microscope. We therefore defined the image obtained from non-FCI animals (Fig. 6D) to represent background oh8G/oh8dG immunoreactivity (oh8G/oh8dG-negative), which we then compared with the images from the FCIR animals. As shown in Table 2, we found a significant increase ($P \leq 0.001$) of the oh8G/oh8dG signal in 17

right ipsilateral cortices of the 19 FCIR animals that experienced 30, 60, or 90 min of FCI and ≤ 30 min of reperfusion. One of the 19 FCIR animals was oh8G/oh8dG-positive in both the right and left cortices, and the other animal was oh8G/oh8dG-negative.

Treatments with DNase-free RNase A (25 $\mu\text{g}/\text{ml}$), which digested tRNA (20 μg) but not DNA (1 μg) in test tubes (37°C, 1 h), drastically reduced but did not abolish the fluorescent signal in the brains that received 60 min of FCI (Fig. 8). The density of oh8dG-positive cells at this time point was 853 ± 113 per mm^2 and was not significantly different from the density of FPGSS-positive cells. We also compared oh8dG immunoreactivity using a previously established IgG antibody. Almost all of the cells in the ischemic cortex from animals with 60/15 FCIR were positive for oh8dG immunoreactivity (Fig. 9). In addition, we observed a positive oh8dG signal in cells (arrows, Fig. 9) that were non-neuronal origins (GFAP-positive, see below). Figure 10 shows GFAP-positive cells (arrows) and GFAP-negative cells (asterisks) exhibiting a trace of oh8dG-immunoreactivity in the right cortex of a control animal and elevated oh8dG-immunoreactivity in the ipsilateral cortex of an FCIR animal (90/01). The green signal expanded mostly in neurons immediately after FCIR. The data suggested that both astrocytes and neurons were vulnerable to DNA damage by hydroxyl radicals during early reperfusion.

FCIR-induced DNA fragmentation

To test whether the DNA damage detected using our FPGSS assay might include DNA fragmentation, TUNEL staining was used to detect DNA strand breaks, including the 3'OH termini of SSB and all forms of DSB (36). Tissue was analyzed at 60 min and at 24 h of reperfusion after 90 min of FCI and at 1–4 days of reperfusion after 30 min of FCI (1,41). We did not observe TUNEL-positive staining during the first 60 min of reperfusion after 90 min of FCI (Table 3). TUNEL-positive staining in brain tissue became apparent in four out of four animals at 24 h of reperfusion after 90 min of FCI. In animals treated with 30 min of FCI, TUNEL-positive brain tissue was apparent beginning on the 2nd day and was significantly increased on the 3rd and 4th days. The nuclei in the ischemic core became condensed under hematoxylin-eosin staining (Fig. 11A), and some of these cells showed TUNEL-positive staining (Fig. 11B), suggesting positive TUNEL staining was a sign of ischemic cell death (1). Most of the cells that were TUNEL-positive were not GFAP-positive (Fig. 11B–D).

DISCUSSION

Excessive oxidative stress causes an elevation in hydroxyl radicals in vulnerable populations of brain cells and may be a key mechanism underlying a number of neurological disorders (43–45). To understand the mechanism that repairs oxidative DNA damage in the central nervous systems, we investigated DNA damage and repair after transient cerebral ischemia. The work we present here suggests that there is a temporal difference between oxidative DNA damage measured using FPGSS and DNA fragmentation measured using TUNEL, and that oxidative DNA damage in the mitochondrial and nuclear DNA of astrocytes and neurons occurs primarily during the first 15 min of reperfusion after cerebral focal ischemia. SSBs bearing 3'-OH termini became apparent ~ 30 min after reperfusion. Our results suggest that the DNA repair process was able to remove the majority of the oxidative DNA damage by 60 min of reperfusion (10). Although the DNA repair process reduced oxidative DNA damage (21), neurons appear more prone to programmed cell death after focal cerebral ischemia than did astrocytes.

Experimental brain injury of the ischemia-reperfusion type, which approximates the injury seen in most stroke patients, affects oxygen supply and perturbs the energy metabolism of the brain (energy failure). The sudden increase in blood supply during reperfusion, as occurs after removal of a vessel block in thrombotic stroke, causes an electron imbalance and results in an

increase in oxygen free radicals in brain cells (20,46). This oxidative stress and the resultant increase in oxygen free radicals may cause various types of RNA/DNA damage, such as strand breaks and base lesions, in addition to causing damage to various proteins (nitrotyrosine) and lipids. Two types of DNA injury induced by oxidative stress have been demonstrated after cerebral ischemia-reperfusion. The first type, DNA fragmentation (14), is the most commonly studied and is found during cell death. Evidence for enzymes that repair DNA fragmentation during cell death is lacking, and, therefore, this type of DNA injury is believed to be irreversible once it starts (12-15). The second type is oxidative DNA damage, which results from attacks on nucleic acids by excess electrons and is often observed after cerebral ischemia (9-11), or ionizing radiation (20,47). This type of damage could also be associated with changing the expression of genes (9,10,48-50). Changing the expression of genes may lead to the initiation of various neurological diseases by mutations or cell death (51). *In vitro*, at least 70 different types of oxidative damage in RNA and DNA can be generated. Eight of these have been identified in the ischemic brain (9-11,15). One of the eight identifiable oxidative DNA lesions in the brain is oh8dG. Oxidative DNA damage in the brain cannot be detected using DNA laddering in agarose gel electrophoresis or using TUNEL staining. Although chromatographic techniques or immunoreactivity can detect oxidative DNA damage, the efficiency of the DNA repair mechanism has often led to conflicting results using these techniques (14,20,24). A reversal of oxidative DNA damage after cerebral ischemia and reperfusion in mice reduces cell death (11). Under normal physiological conditions, the repair of oxidative DNA damage is generally mediated by DNA repair processes (24). The consensus belief is that DNA repair processes are generally beneficial to the cell during normal metabolism. After stroke or traumatic brain injury, when energy becomes critical to the viability of the affected tissue, certain repair steps, such as the ligation step that involves DNA ligase and poly(ADP-ribose) polymerase (PARP), may deplete further the needed energy source and may be harmful to the very tissue that the repair process is attempting to rescue. Therefore, there is an urgent need to delineate the mechanism of oxidative DNA damage and repair in the brain.

We have shown here that oh8G/oh8dG lesions, detected *in situ* as immunoreactivity to newly developed monoclonal 8G-14 IgM, or as substrates to *E. coli* Fpg protein, appear in brain cells of the affected cortex within the first 30 min of reperfusion. The results using 8G-14 IgM agree with those from the more commonly used chromatographic methods (9,23,45). The 8G-14 IgM antibody specifically binds to oh8G-BSA and gives a positive cytosolic signal that is comparable to the results described previously using different antibodies (10,29). Several assays that detect DNA damage in the brain have been reported (9-11,29,30). The current method offers several additional advantages. First, we were able to show that oxidative DNA damage occurs in mtDNA and nuclear DNA, because immunoreactivity of cytoplasmic oh8dG to 8G-14 IgM antibody in the FCIR cortex did not disappear after treatment with RNase A. Our results are in contrast to those found in patients with Alzheimer's disease, and the difference could be in the concentration of RNase A used by Nunomura et al. (29). Second, we were able to show that although both neurons and astrocytes contain oxidative DNA damage early during reperfusion, the only cells in the ischemic core that showed signs of cell death were neurons.

Our detection of cellular oh8G/oh8dG immunoreactivity using 8G-14 IgM and IgG confirms our FPGSS findings, except that we observed more cytosolic lesions using the immunohistochemical method than using the *in situ* FPGSS method. The discrepancies may be in the assays that were used. Cellular IgM or IgG immunoreactivity labels oh8G in RNA and oh8dG in mtDNA (10), whereas the FPGSS assay labels other oxidative base damage in both mtDNA and nuclear DNA. The current study supports the notion that mRNA and its nuclear gene could be targeted by oxygen free radicals during reperfusion (9-11). We have previously estimated that the frequency of FPGSS on the genomic level is approximately two FPGSS per reporter gene (9-11,21). Our observation that neurons are more prone to cell death

than our astrocytes after FCIR in this rat model agrees with our previous observations using the mouse forebrain ischemia and reperfusion model (11). Evidence from other studies suggests that astrocytes repair oxidative DNA damage with a greater efficiency than do neurons (47).

In addition to base modifications, oxidative DNA damage also includes DNA strand breaks (11,15,52,53). Three mechanisms are known to produce DNA strand breaks: 1) an attack on DNA by hydroxyl radicals (9-11,20), 2) the digestion of base modification by DNA lesion-specific enzymes (21,65,66), and 3) an attack on DNA by nonspecific nucleases during programmed cell death (15). Our inability to identify significant SSB/5'DSB at or before 15 min of reperfusion does not exclude the possibility that FCIR induces other types of DNA strand breaks with 3'phosphoglycolate termini (53), which cannot be detected using the FPGSS assay. The end-labeling assay by DNA polymerase-I in the absence of Fpg protein indicated the presence of SSB/5'DSB bearing 3'-OH termini beginning at 30 min of reperfusion (10, 11). Moreover, we did not observe TUNEL-positive staining at <120 min of reperfusion, suggesting that the DNA strand breaks we detected at 30 – 60 min of reperfusion were not a result of apoptosis-associated DNA fragmentation. Our studies suggest that a temporal sequence of oxidative stress-induced DNA damage starts with the beginning of reperfusion—oxidative DNA base modifications, followed by the formation of SSB/5'DSB, followed by DNA fragmentation and cell death.

The appearance of these SSB/5'DSB with 3'-OH ends could be the intermediates of the repair activity (9-11,21,52,54). Because DNA strand breaks are known to activate PARP, our data would predict that the activation of PARP could be at or after 30 min of reperfusion after cerebral injury. Indeed, most studies suggested that PARP is activated at least 30 min after brain injury (55). An elevation in reactive oxygen species may be an etiological factor in Alzheimer's disease (9,17,29,56), Parkinson's diseases (44), Batten's disease (57), seizure disorders (58), stroke (59), ischemia (9-11,15,17,21,60), Hallervorden-Spatz disease (61), amyotrophic lateral sclerosis (62,63), and brain edema (64). Further studies are needed to determine whether there is an elevation in oxidative stress and to measure the ability of these patients to repair DNA damage.

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Figure 1.

The ability of *E. coli* DNA polymerase-I (Kornberg pol-I) to extend synthesis of the 3'-end generated by *E. coli* Fpg protein. A synthetic DNA sequence of *c-fos* gene (5'-CATCATGGTCZTGGTTTGGGCA-3', where Z is the oh8dG) was labeled on the 5' end using [γ]- 32 P-ATP (21), then was hybridized to the complementary strand, which C was opposite Z. The double-stranded DNA (7.5×10^6 cpm/pmol, 100 fmol) was treated with buffer (lanes 1, 2) or with Fpg protein (0.15 μ g, lanes 3–10) in 37°C for 10 min, followed by heating at 80°C for 10 min. All of the reaction products were then incubated with dNTP (40 μ M) (lanes 1–10) and additional endonuclease-free DNA polymerase-I (2.5 U, two different preparations of Kornberg enzyme [lanes 5, 6, 9, 10] or endonuclease-free Klenow enzyme [lanes 7, 8]) at 37°C for 5 min. The reaction was stopped by heating and then resolved in 10% sequencing PAGE gel to analyze single-strand DNA.

Figure 2. FPGSS in the cortex after FCIR. FPGSS in the left (A) and the right (B) cortices from a Long-Evans rat treated with FCIR (90/15). FPGSS appear as the white fluorescent signal. Bar = 20 μm .

Figure 3.

The incorporation of dig-dUTP by Kornberg DNA polymerase-I is dependent on SSB bearing 3'-OH ends. The incorporation of dig-dUTP to 3'-OH termini (white fluorescent signal) in the sham-operated control brains (one of four is presented here) was tested using only Fpg protein (A), Kornberg DNA polymerase-I (B), or DNase I and then Kornberg DNA polymerase-I (C). The fluorescent signal that can be observed in panels A and B comes mostly from the background of the cytoplasm. Similar results were noted in another set of experiments when Kornberg enzyme was replaced with Klenow or terminal transferase (not shown). Bar = 35 μm .

Figure 4.

FPGSS in rat brain after FCI and reperfusion. Four representative ipsilateral cortices from Table 2 show FPGSS in sham-operated animals and animals treated with FCI (90 min) and reperfusion of various time intervals. The tissue in the left panels (A—D) was treated with buffer and the Kornberg polymerase-I only; the tissue in panels E—H was treated with Fpg protein then the Kornberg polymerase-I. The green fluorescent signal indicates the FPGSS and is the strongest at 15 min of reperfusion (90/15) and at 90/30 min of reperfusion. Perinuclear signals (asterisks) are shown in the 90/60 groups. At 30 and 60 min of reperfusion, some signal appears in tissue without Fpg protein (non-FPGSS or SSB/5'DSB, arrows). Bar = 40 μ m.

Figure 5.

8-OH-G antigen in the cortex after FCI-reperfusion. The 8G-14 IgM antibody was used to demonstrate the presence of oxidative DNA lesions after transient ischemia. The figure shows the right hemisphere surrounding the lateral ventricle at low magnification. The green fluorescence indicates the presence of the FITC-antibody-antigen complex; the background was stained yellow by the fluorescent antifade-mounting medium (Sigma, two particulates are present in the image). The cortical samples are from one of three animals treated with 90 min of FCI and 15 min of reperfusion. A higher magnification is shown in Fig. 6. Bar = 100 μ m.

Figure 6.

Immunoreactivity of oh8G/oh8dG is stronger in cytosol than in nuclei after FCIR. A higher magnification of typical ipsilateral brain tissue from each group of animals with 90/15 FCIR (A—C, E, F) or without FCIR (D) are shown: A, D—F) frontal cortex; B) parietal cortex; C) corpus callosum. The smaller patch of green signal could be from overlapping astrocytes. The IgM antibody in panels E, F was either preadsorbed with the 8-OH-g-BSA conjugates (50 ng, E) or deleted in the assay (F), and they serve as the negative controls. The FITC signal was strong enough that preadsorption did not completely eliminate the green FITC signal in panel E. Arrows show signals in the cytoplasm and perhaps nuclei such that the intensity of yellow antifade was reduced. Bar = 10 μ m.

Figure 7. Specificity of 8G-14-antibody binding for the oh8G-BSA conjugate using radioimmunoassay (see Materials and Methods). Four determinations in two separate experiments were performed. The mean (means) and sd (bars) at each dilution are shown.

Figure 8.

The immunoreactivity of oh8dG (IgM-8G-14) remains visible after RNase treatment. A typical image of the right cerebral cortices from one of three animals underwent FCIR (60/0) and RNase A (25 μ g/ml, 30 min at room temperature, bottom panel) treatment before the addition of the 8G-14 IgM. The green fluorescent signal was visible in area surrounding the nuclei.

Figure 9.

Immunoreactivity of oh8dG using commercially available IgG and pretreatment of RNase A. The FCIR cortex after 60 min of FCI ($n=3$) is shown. Tissue was pretreated with RNase A before the IgG antibody against oh8G/oh8dG. Arrow shows strong oh8dG-immunoreactivity in the location where GFAP-positive cells occupied (see Fig. 11). Stars show the rims around the nuclei, a possible indication of mitochondrial DNA damage (see Fig. 8). Bar = 65 μ m.

Figure 10.

GFAP-positive and GFAP-negative cells contain oh8dG-immunoreactivity. This figure shows the double-stain for GFAP- (top panels) and oh8dG- (bottom panels) immunoreactivity. Arrows indicate the location of GFAP-positive cells; asterisks indicate location of GFAP-negative cells. A) sham-operated control; B) 90/01 of FCIR. Bar = 10 μ m.

Figure 11.

TUNEL-positive stain after FCIR. Coronal sections with HE stain (A) or with double staining for TUNEL and GFAP immunoreactivity (B—D) in the right cerebral cortex (the ischemic core and border area surrounding the core) from one of four typical animals with 4 day reperfusion after 30 min FCIR. Green fluorescent: TUNEL staining; red fluorescent: astrocytes. Asterisks: neurons at different magnifications; arrows: GFPA-positive cells in area where oh8dG-(FPGSS-)positive cells were observed (Fig. 9, 4G). Bar = 50 μ m (A, B) or 10 μ m (C, D).

TABLE 1

FPGSS and strand breaks in the ipsilateral cortex of rats treated with FCI

Treatments	n	FPGSS negative	FPGSS positive ^a	Non-FPGSS positive ^b
Non-FCI (control)	9	7	1	1
Reperfusion time (min) after 90 min FCI				
1	6	0	5	1
15	5	1	3	1
30	4	0	3	1
45	2	0	2	0
60	7	0	6	1
120	4	0	1	3 [*]
FCI total	28 [*]	1	20	7

^aFPGSS-positive animals were those having higher FPGSS in the right cortex than in the left cortex (see Materials and Methods).

^bWhen positive cells occur in animals without prior treatment of Fpg protein, we defined the animals positive in non-FPGSS signals (single-strand breaks or double-strand breaks with protruding 5' ends [SSB/5'DSB]).

* $P \leq 0.05$ by Fisher's Exact test compared with control animals.

TABLE 2

The immunoreactivity of oh8G/dG in the ipsilateral cortex after FCI

Treatments	n	oh8G/oh8dG-positive ^a		
		No	Yes R	Yes R/L
Non-FCI (control)	5	5	0	0
Reperfusion of ≤ 30 min after FCI of				
30 min	6	0	6	0
60 min	3	1	2	0
90 min	10	0	9	1
FCI total	19 [*]	1	17	1

^a See Materials and Methods for definition of 8-OH-G-positive animals. R; the right cortex only, R/L the right and left cortices.

^{*} $P \leq 0.001$ by Fisher's Exact test compared with control animals.

TABLE 3

DNA fragmentation as determined by the TUNEL assay

Treatment	n	TUNEL negative	TUNEL positive ^a
90 min of FCI +			
<2 h of reperfusion	6	6	0
24 h of reperfusion	4	0	4
30 minutes FCI + reperfusion (days)			
1	4	4	0
2	4	2	2
3	4	1	3 [*]
4	4	0	4 ^{**}

^a TUNEL-positive animals are defined in Materials and Methods.

^{*} $P \leq 0.05$

^{**} $P \leq 0.005$ by Fisher's Exact test compared with control animals.