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Increased mitochondrial DNA oxidative damage after transient middle cerebral artery occlusion in mice

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Abstract

Oxidative stress and DNA oxidation play important roles in the induction of ischemic neuronal cell death. However, the subcellular source of oxidized DNA detected by 8-hydroxy-2′-deoxyguanosine (8-OHdG) after ischemia has not been clarified although it is known to increase in the brain after ischemia. One-hour transient ischemia of the middle cerebral artery was induced in mice utilizing an intraluminal filament. The occurrence of superoxide anion as an ethidium (Et) signal, 8-OHdG, cytochrome *c* release and neuronal cell death were examined using immunohistological and biochemical techniques in sham-operated control (0 h) and 1, 3, 6, 24, or 96 h after reperfusion. Et signals were prominent in the cortical neurons of ipsilateral hemisphere 3 h after reperfusion. Strong 8-OHdG immunoreactivity was observed 3–6 h after reperfusion. Immunoassays after cell fractionation revealed a significant increase of 8-OHdG in mitochondria 6 h after reperfusion. Immunohistochemistry revealed that the 8-OHdG immunoreactivity colocalized with a neuronal marker, microfilament 200 and a mitochondrial marker, cytochrome oxidase subunit I. Cytochrome *c* rose in cytoplasm at 6 h and TUNEL-positive neurons noted 6–24 h after ischemia. The present results suggest the possibility that the mitochondrial damage including mitochondrial DNA oxidation might be responsible for the induction of ischemic neuronal cell death.

Keywords: 8-Hydroxy-2′-deoxyguanosine (8-OHdG); Transient focal ischemia; Mitochondria; DNA oxidative damage; Cytochrome *c*; Neuronal cell death

1. Introduction

An increase in reactive oxygen species (ROS) has been indicated as one of the earliest events after ischemia/reperfusion. Recently, several studies have shown a pivotal role of the oxidative stress or damage in the induction of neuronal cell death after ischemia (Fujimura et al., 2005; Moro et al., 2005; Skulachev, 1999). The ROS react with intracellular proteins, lipids, sugars and nucleotides and impair the normal function and physiology of cells.

8-Hydroxy-2′-deoxyguanosine (8-OHdG) is an oxidized form of deoxyguanosine, a component of DNA, and is utilized as a DNA oxidation marker. Under normal conditions, the oxidized DNA can be repaired *in vivo* by endonucleases, or by a base-specific glycosylase and the 8-OHdG is transported through the blood and excreted into the urine without further modification. However, in pathological conditions such as cancer, inflammation, diabetes and neurodegeneration, 8-OHdG levels increase in tissues, blood or urine for the oxidative stress. Brain levels of 8-OHdG are increased in aging (Fraga et al., 1990) and in animal models and patients with Huntington's disease (HD) (Bogdanov et al., 2001; Hersch et al., 2006), ischemia (Wakatsuki et al., 1999; Nagayama et al., 2000; Ohtaki et al., 2003b; Zhang et al., 2005), amyotrophic lateral sclerosis (ALS) (Ferrante et al., 1997; Aguirre et al., 2005), Parkinson's disease (PD) (Sato et al., 2005), Alzheimer's disease (AD) (Mecocci et al., 1994; Aliev et al., 2003), and multiple sclerosis (Vladimirova et al., 1998). It has been reported that 8-OHdG level increases in mitochondria due to the single strand DNA (mtDNA) which makes it more

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susceptible to damage (Bogdanov et al., 2001; Aliev et al., 2003; Shimura-Miura et al., 1999; De la Monte et al., 2000; Warita et al., 2001; Iida et al., 2002).

Mitochondria are critical for the production of ATP as the energy of cells via Krebs cycle and electron transport system. Mitochondria are involved in the regulation of cytosolic Ca^{2+} levels, intracellular pH, the induction of apoptotic-like cell death, and are the major source of ROS. Therefore, this organelle also plays a pivotal role of the processes on neuronal cell death (Chan, 2004; Foster et al., 2006; Sims and Anderson, 2002).

However, the subcellular source of the oxidized DNA detected by 8-OHdG staining in the brain after ischemia is unknown. In this study, we investigated the time-dependent occurrence of oxidative damage in mitochondria after ischemia using immunohistochemical and biochemical techniques.

2. Materials and methods

2.1. Animals

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Showa University (#03019). Male adult C57/B6J mice were purchased from Charles River (Tokyo, Japan). Animals were maintained under conditions of a 12 h light/dark cycle and had free access to food and water.

2.2. Antibodies and reagents

Mouse monoclonal anti-8-OHdG antibody was obtained from NOF Corporation (Tokyo, Japan). Alexa 488-conjugated anti-cytochrome oxidase subunit I (COX; a mitochondrial marker), Alexa-labeled secondary antibodies, and hydroethidium (HEt), used for the *in situ* detection of superoxide anion (O_2^-), were obtained from Molecular Probes (Eugene, OR). Rabbit polyclonal anti-neurofilament 200 (NF200, Sigma, St. Louis, MO) and mouse monoclonal anti-NeuN antibody (Chemicon, Temecula, CA) were used as neuronal markers. Rabbit polyclonal anti-cytochrome *c* and mouse monoclonal anti-α-tubulin were obtained from Santa Cruz (Santa Cruz, CA) and Sigma, respectively. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG were obtained from Amersham (Piscataway, NJ). 4′,6-Diamidine-2-phenylindole dihydrochloride (DAPI) and *In Situ* Cell Death Detection Kit, POD as a terminal deoxynucleotidyl transferase-mediated dUTP end-labeling (TUNEL) were obtained from Roche (Manheim, Germany).

2.3. Transient middle cerebral artery occlusion (tMCAO)

Ischemic insult in mice was induced according to an intraluminal technique as described previously (Ohtaki et al., 2003a, 2006). Briefly, the mice (24–29 g) were anesthetized by inhalation of N_2O/O_2 mixture (2:1) with 3.0% sevoflurane through a facemask and body temperature was maintained at 37.0–38.0 °C by heat blanket. A 7-0 round tip nylon suture was introduced into the internal carotid artery and advanced 8–10-mm distal to the carotid bifurcation to occlude the middle cerebral artery (MCA). One hour after induction of ischemia, the animals were reperfused by the withdrawing the suture. During ischemia and for a few hours after reperfusion, the animals were kept under the heat lump to prevent hypothermia. Animals that did not show any neurological deficit (Hara et al., 1996; Ohtaki et al., 2006) 1 h after ischemia, immediately before reperfusion, or those that hemorrhaged were excluded from the experiment.

2.4. In situ detection of O_2^-

Production of O_2^- in the ischemic brain was determined by *in situ* detection of oxidized HEt (Bindokas et al., 1996; Murakami et al., 1998). The HEt rapidly penetrates the brain parenchyma, reacts with O_2^-, and generates ethidium (Et) which is detected at an emission wavelength of 510–550 nm. Mice subjected to tMCAO were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.) at 0 h (sham-operated control), 1 h or 3 h and were administered HEt solution (1 mg/mL 0.9% NaCl with 1% DMSO) into the jugular vein ($n = 3$ per condition). Fifteen minutes after the administration, the animals were perfused with 2% paraformaldehyde (PFA) and the brains were then removed and postfixed in 2% PFA. The brains were then cryoprotected in 20% sucrose, frozen in liquid nitrogen-cooled 2-methylbutane and cryosectioned (8 μm) in the coronal plane. To demonstrate the distribution of Et signals in sections, the sections were co-stained with NeuN. After blocking with 2.5% normal horse serum (NHS), the sections were incubated with mouse anti-NeuN (1:1000) antibody and were visualized by Alexa 488-labeled anti-mouse IgG (1:400). All of the above procedures were carried out on three animals per condition.

2.5. Immunostaining of 8-OHdG

After anesthetizing deeply, mice 0, 1, 3, 6, 12, 24, or 96 h after tMCAO were perfused and their brains processed and sectioned as described above. The sections were blocked with 2.5% NHS for 1 h and incubated with mouse anti-8-OHdG antibody (1:100). After washing, the sections were incubated with Alexa546-labeled goat anti-mouse IgG (1:400) and were counterstained with DAPI (1:10,000). To identify the intracellular localization of 8-OHdG, some sections were incubated additionally with Alexa 488-labeled mouse anti-COX antibody (1:500). Controls included the omission of the primary antibody for all immunostaining.

The number of 8-OHdG-positive cells was semiquantified on the basis of DAPI-staining in three different regions: the somatosensory (SC) and piriform cortices (PC) and the striatum (ST) in a coronal section (between +1.10 and −0.14 mm of bregma) of each hemisphere. After taking images of 8-OHdG- and DAPI-staining in same region under a 40× objective, the contrast was enhanced, and the number of DAPI-stained nuclei was counted to obtain the total number of cells. The 8-OHdG-positive cell number was then counted on the basis of the position of DAPI-stained nuclei. The area of the image in the region under investigation was 820.5 μm × 615.4 μm, and the number of 8-OHdG-positive cells expressed as a percentage of the total number of cells. Evaluations were performed on sections obtained from the brains of three to six mice at each time point. An investigator who was unaware of the regions, time and hemisphere quantified the number of 8-OHdG-positive cells.

2.6. Enzyme immunoassay of 8-OHdG

Telencephalons were removed under anesthesia 0, 1, 3 or 6 h after tMCAO and divided into the ipsilateral and contralateral hemispheres ($n = 3$–4). The samples were fractionated immediately using a Mitochondria/Cytosol Fractionation Kit (BioVision Research Products, Mountain View, CA). Briefly, the samples were homogenized in 1 mL of cytosol extraction buffer with 30 strokes of a Dounce homogenizer. The homogenates were centrifuged twice at 700 × *g* for 10 min to precipitate the nuclear fraction. The supernatant was then centrifuged at 10,000 × *g* for 30 min and the pellets and supernatants were collected as the mitochondrial and cytoplasmic fractions, respectively.

Genomic and mitochondrial DNAs were isolated using a NucleoSpin Tissue kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions and content and purity were measured spectrophotometrically. The DNAs were hydrolyzed with nuclease P1 (Yamasa Corp., Choshi, Japan) in 20 mM sodium acetate (pH 4.8) at 37 °C for 30 min and with alkaline phosphatase (Oriental Yeast, Suita, Japan) in 0.1 M Tris/HCl (pH 7.4) at 37 °C for 60 min. Samples were filtered using Ultrafree MC (Millipore, Billerica, MA) at 14,000 rpm for 10 min to remove hydroxylases (Kakimoto et al., 2002). The 8-OHdG content (ng/μg DNA) in the hydrolyzed genomic and mitochondrial DNAs were measured using Highly Sensitive 8-OHdG ELISA kit (JaICA, Furkuroi, Japan) according to the instruction manual.

2.7. Cytochrome c release from mitochondria

For multiple staining, the brain sections obtained from mice 0, 6, or 24 h after tMCAO were incubated with anti-cytochrome *c* antibody (1:500) after blocking ($n = 3$ per time point). The sections were then incubated with Alexa 546-labeled anti-rabbit antibody. The sections were subsequently incubated for 2 h with Alexa 488-labeled anti-COX antibody.