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Effects of one-day reperfusion after transient forebrain ischemia on circulatory system in the rat

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Abstract. Although ischemia/reperfusion injury remains incompletely understood, it appears that reactive oxygen species produced mainly during postischemic recirculation play a critical role. The present study examined the impact of forebrain ischemia and subsequent one-day reperfusion on several blood parameters. We determined glutamate concentration in whole blood, measured Cu/Zn- and Mn-SOD (superoxide dismutase) activity in blood cells as well as plasma, and investigated the prevalence of single and double strand breaks of lymphocyte DNA. The results of our experiment showed that the concentration of glutamic acid in whole blood was increased by about 25%. Antioxidant activity of total SOD and Cu/Zn-SOD was reduced in blood cells and plasma. Mn-SOD activity in blood cells was not affected by ischemic insult and one-day reperfusion, but we detected its significantly lower activity in samples of plasma. We observed a weakly reduced level of double and a significantly elevated level of single strand breaks of lymphocyte DNA.

In conclusion, one day of recovery after the ischemic attack failed to return peripheral circulatory system to physiological conditions. Reduced antioxidant capacity in the blood and an elevated level of excitotoxic amino acid glutamate may cause lymphocyte DNA damage, and probably contribute to insufficient postischemic recovery of brain tissue.

Key words: Ischemia — Lymphocyte — Blood — Glutamate — Superoxide dismutase

Abbreviations: BSA, bovine serum albumin; DSBs, double strand breaks; EDTA, ethylenediaminetetraacetic acid; NBT, p-nitrotetrazolium blau grade III; PBS, phosphate buffered saline; ROS, reactive oxygen species; SCGE, single cell gel electrophoresis; SHC, sham control sample; SOD, superoxide dismutase; SSBs, single strand breaks; TAE, tris-acetate-EDTA buffer; U, unit of enzyme activity.

Introduction

Ischemic conditions trigger several detrimental events in the brain tissue, which dictate the resulting brain damage. Energy imbalance, disrupted ion (Ca^{2+}) homeostasis and extensive release of excitatory transmitters, mainly glutamic acid, are the front line mediators of tissue damage.

Glutamate is a major excitotoxic amino acid that occurs naturally in mammalian central nervous system. Under physiological conditions, glutamate is responsible for neurotransmission through activation of ionotropic and metabotropic glutamate receptors (Meldrum 2000). Brain ischemia leads to massive release of glutamic acid from cells to extracellular space. This increased level of extracellular glutamate may induce oxidative stress (Michaels and Rothman 1990) that leads to a reduced ability to protect against oxidative injury of the cell, resulting in the production of free radicals, inflammation and apoptosis (Rosamond et al. 2008).

All aerobic cells have enzymatic systems for protection against reactive oxygen species (ROS). The enzymatic defense against activated oxygen species involves co-operative action of three main intracellular antioxidant enzymes: superoxide dismutase (SOD), present in the cytosol, and glutathione

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peroxidase and catalase, present in peroxisomes (Michiels et al. 1994).

SOD is an endogenous antioxidant enzyme involved in the detoxification of superoxide radicals. Three natural SOD isozymes were described (Marklund 1982). Intracellular Cu/Zn-SOD and Mn-SOD isozymes are located in the cytosol and in the mitochondrial matrix, respectively. Both the Cu/Zn-SOD and Mn-SOD isoenzymes have been shown to play important roles in the defense against ischemic brain damage (Li et al. 1998; Kawase et al. 1999; Danielisova et al. 2005).

Important sources of antioxidant enzymes in blood circulation are blood cells (Scott et al. 1989; Pietarinen-Runtti et al. 2000). The blood level of ROS and agents inducing cell production of ROS are elevated after brain ischemia (Leker and Shohami 2002), and blood cells are sensitive to higher oxidative stress (Priezzhev et al. 2004; Sivonova et al. 2008). For example, hypoxia reoxygenation treatment of human lymphocytes induced rapid ROS generation and mitochondrial membrane potential collapse, which triggers ROS caspase-8-mediated apoptosis of lymphocytes (Choi et al. 2007; Kim and Chung 2007).

Recently, some authors have pointed out the relevance of the presence of SOD enzyme in blood cells and plasma of stroke patients (Spranger et al. 1997; Alexandrova et al. 2004; Cherubini et al. 2005), suggesting the possibility of red blood cells serving as a carrier of SOD to scavenge O^{2-} produced during cerebral ischemia and reperfusion (Wang et al. 1996, 1997).

The most important requirement for the survival of affected brain tissue is blood supply restoration. At the same time, recirculation may be a source of ROS produced by natural immune response. Stroke is followed by an acute and a prolonged inflammatory response (Wang et al. 2007) characterized by the production of inflammatory cytokines (Pasarica et al. 2005; Frangogiannis 2007) leukocyte and monocyte infiltration in the brain (Stroh et al. 2006), and the activation of resident glial cells (Rischke and Krieglstein 1991; Gehrmann et al. 1992; Beschorner et al. 2007). These events may affect the ischemic brain in a positive way, although several groups report conflicting results regarding the role of inflammation and the effects of anti-inflammatory treatments in cerebral ischemia (Turner and Vink 2007).

Our previous data (Kravcukova et al. 2009) document the effects of ischemia/reperfusion injury on the circulatory system in the early stages of recirculation. In the present work we studied the impact of postischemic conditions on blood plasma and cells one day after the ischemic insult. We investigated changes in the level of glutamic acid in whole blood, alterations in the activity of SOD in plasma and blood cells (total SOD, Cu/Zn-SOD and Mn-SOD) and finally, the effect of postischemic condition on circulating cells (range of DNA damage of peripheral blood lymphocytes).

Materials and Methods

Model of transient forebrain ischemia

Experiments were approved by the Institutional Ethical Committee, in accordance with current national legislation. All efforts were made to minimize animal suffering and to reduce the number of animals used. Adult male albino *Wistar* rats ($n = 8$) weighing 250–300 g were maintained on a 12 h light/dark cycle and given food and water *ad libitum*. Food was withdrawn one day before surgery.

The animals were subjected to a four-vessel occlusion model of transient forebrain global ischemia (Pulsinelli and Brierley 1979) with modifications (Schmidt-Kastner et al. 1989). The rats were anesthetized with 2.5% halothane and maintained during surgery at 1.5%. On the first day, both vertebral arteries were irreversibly occluded by coagulation through the alar foramina. The next day, rats were reanesthetized and the common carotid arteries were clamped by small atraumatic clips to induce forebrain ischemia. Rats that did not become unresponsive within 60 s after clasp tightening were excluded from the study. Criteria for ischemia were loss of the righting reflex, paw extension, and mydriasis. During ischemia, animals were kept in the recumbent position and physiologic body temperature was maintained by a heating pad. After 10 min, the carotids were released and restoration of carotid artery blood flow was verified visually.

For histological confirmation of ischemic conditions, the rats survived for 7 days. Hippocampal CA1 region neurodegeneration was visualized using FluoroJade B staining (Schmued and Hopkins 2000) (Fig. 1).

Blood collection and sample preparation

Samples of whole blood (approx. 500 µl) were obtained by cannulation of right external jugular vein to the right atrium durinxg ischemia induction. Sham control sample (SHC) was collected directly before bilateral carotid occlusion, test samples were collected one day after restoration of blood circulation to the brain.

Blood aliquots for the measurement of glutamate levels (70 µl) were deproteinized by adding an equal volume of ice-cold 1 mol/l perchloric acid (Fluka), left on ice to precipitate for 15 min and then centrifuged at 10 000 × *g* for 10 min at 4°C. The pellet was discarded and the supernatant was collected and stored at –80°C for later analysis.

SOD activity was measured in plasma and blood cell lysates of collected blood samples. Plasma was obtained by centrifugation (10 000 × *g*, 4°C, 10 min). Pellet was resuspended in distilled water in a volume equivalent to the volume of collected plasma. After vortexing, cell suspension was rapidly frozen (10 min, –20°C) and defrosted at 37°C.

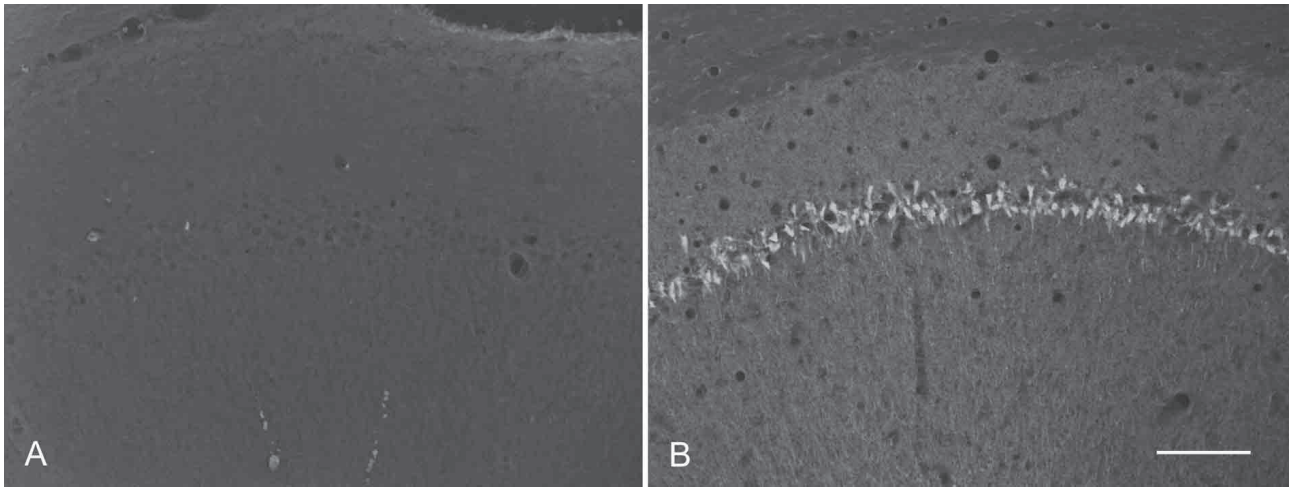

Figure 1. Representative photomicrographs of neurodegeneration in the hippocampal CA1 region visualized by Fluoro Jade B staining. **A.** Sham control animal. **B.** Animal with 10 min of ischemia and 7 days of recovery. Scale bar: 100 μm.

This procedure was repeated twice and homogenized pellet was finally centrifuged (10 000 × *g*, 4°C, 10 min). Supernatant representing blood cell lysate was stored at –80°C until analysis.

Lymphocytes were isolated from 200 μl of whole blood diluted in phosphate buffered saline (PBS; Sigma; pH 7.4, 4°C, dilution 1 : 4) by density centrifugation (2500 rpm, 5 min, 4°C) on Ficoll-Paque^{TM} Plus gradient (Amersham Pharmacia Biotech. AB, Sweden). Lymphocyte layer was collected and lymphocytes were rewashed in PBS (1 : 4) in the same conditions and directly used for DNA damage assessment (single cell gel electrophoresis).

Only blood samples of animals with over 70% neurodegeneration in the CA1 hippocampal region were included in the experiments.

Blood level of glutamic acid

Whole blood glutamate level (μmol/l) was measured by enzymatic-fluorimetric method described by Graham and Aprison (1966) with modifications for microplate reader (Kravcukova et al. 2009). Glutamate concentration in the samples was determined by fluorometric detection of NADH resulting from the reaction of glutamate and NAD^+ catalyzed by glutamate dehydrogenase. Glutamate concentration is directly proportional to the concentration of NADH in a reaction. Briefly, 10 μl of supernatant was mixed with 190 μl of reaction buffer (0.25 mol/l hydrazine hydrate/0.3 mol/l glycine buffer, pH 8.6) containing 200 nmol/l NAD^+ and 15 U of glutamate dehydrogenase. After 30 min of incubation at room temperature, fluorescenece intensity of final product NADH was read in a Synergy™ 2 Multi-mode microplate reader (BioTek) at 460 nm with an excitation wavelength 360 nm. All chemicals used for glutamic acid concentration measurements were purchased from Fluka.

SOD activity

SOD activity assay was based on the indirect inhibition assay developed by Sun and coworkers (1988) and first described by Beauchamp and Fridovich (1971) with modifications for microplate reader (Kravcukova et al. 2009). Xanthine–xanthine oxidase was utilized to generate superoxide flux. The absorbance obtained from nitroblue tetrazolium (NBT, p-nitrotetrazolium blau grade III, Sigma) reduction to blue formazan by superoxide was determined at 560 nm spectrophotometrically at room temperature. SOD in the sample competes for superoxide, inhibiting the rate of reaction of superoxide with NBT. The rate of NBT reduction in the absence of tissue was used as the reference rate (0.020 ± 0.005 absorbance/min). The standard assay substrate mixture contained in 0.2 ml: 1 mol/l xanthine (Sigma), 0.1 mol/l EDTA, 5.6×10^{-2} mol/l NBT and 1 mg/ml BSA (bovine serum albumin, Fluka) in 0.1 mol/l sodium phosphate (pH 7.8). One unit of SOD activity was defined as the amount that reduced the absorbance change by 50%, and results were normalized on the basis of total protein content (U/mg protein). The data were plotted as percentage of inhibition vs. protein concentration. Cu/Zn-SOD was differentiated from Mn-SOD by the addition of 2 mmol/l sodium cyanide to inhibit the activity of Cu/Zn-SOD. Cu/Zn-SOD activity was calculated as the difference between total SOD and Mn-SOD activity (McIntosh et al. 1998). Total protein concentrations in plasma and blood cell samples were determined using the method described by Bradford (1976) and BSA was used to establish a standard curve.

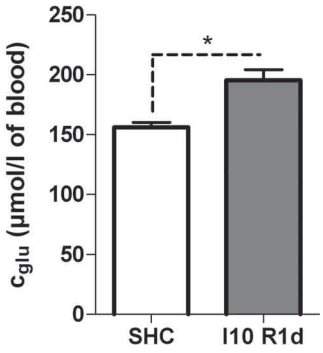

Figure 2. Whole blood glutamate concentration one day after transient cerebral ischemia. Data are expressed as mean ± SD. SHC, sham control; I10R1d, ischemia 10 min/recirculation 1 day; c_{glu}, glutamate concentration (µmol) in liter of whole blood; * $p < 0.001$.

Single Cell Gel Electrophoresis (SCGE)

DNA damage of circulating lymphocytes was measured by a protocol described by Singh and coworkers (1988) with minor modifications. Briefly, 50 µl of lymphocyte suspension mixed with 1% low melting point agarose in PBS (pH 7.4) was added onto microscope slides precoated with 1% normal melting point agarose in PBS (pH 7.4) and allowed to solidify covered with cover-slips in a refrigerator. After solidification of the gel, the cover-slips were removed and the slides were submerged in lysing solution (2.5 mol/l NaCl, 100 mmol/l Na_2EDTA, 10 mmol/l Tris, 1% Triton X-100, pH 10) for 1 h. For the assessment of single strand breaks (SSBs) of lymphocyte DNA (alkaline version of SCGE), the slides were then placed in unwinding buffer (5 mol/l NaOH, 200 mmol/l Na_2EDTA, pH 12.1) for 40 min and electrophoresis was carried out using the same solution for 20 min at 25 V and 300 mA. After electrophoresis, the slides were neutralized with neutralization buffer (400 mmol/l Tris, pH 7.5, 15 min). All previously described steps were carried out in a refrigerator (4°C).

For the assessment of double strand breaks (DSBs) of lymphocyte DNA, we used a neutral version of SCGE (Klaude et al. 1996). Electrophoresis was performed in TAE buffer (40 mmol/l Tris acetate, 1 mmol/l EDTA) at pH 8.0, for 20 min (25 V, 16 mA, 4°C) after a 30 min equilibration period in the same buffer. All chemicals used were purchased from Sigma.

Both types of electrophoretic slides were stained with SYBR Green I nucleic acid gel stain directly before imaging under a fluorescence microscope (Olympus BX51, ex.f. 485, em.f. 520 nm) equipped with a camera (Olympus DP50). Images of comets (software Olympus DP image) were processed using CometScore^{TM} 1.5 image analysis system (TriTek Corp., USA). Lymphocyte DNA damage was examined by parameter "% DNA in tail" (100% of cell fluorescence intensity minus intensity of head % DNA). For each individual sample, two slides were prepared and cells were randomly selected for analysis (total 100 cells *per* sample).

Statistical analysis

Data are expressed as a mean ± SD. Statistical analysis was performed with Student´s *t*-test. The value of $p < 0.05$ was considered to be statistically significant.

Results

Concentration of glutamic acid

The level of glutamic acid in whole blood was increased 24 hours after the ischemic attack (Fig. 2). Glutamate concentration reached 195.5 ± 8.76 µmol/l of whole blood, which was significantly higher by 25.16% than the baseline level represented by SHC (156.2 ± 3.97 µmol/l of whole blood).

SOD activity

Results of our experiment showed that one-day reperfusion after global ischemia leads to a decrease in total and Cu/Zn-SOD activity in plasma as well as in blood cells.

Activity of total SOD of SHC reached 0.64 ± 0.034 U/mg of protein in plasma and 2.122 ± 0.330 U/mg of protein in blood cells, respectively. Twenty four hours after the ischemic insult total SOD activity decreased by about 72% in plasma and by 50% in blood cells. Similarly, Cu/Zn-SOD activity decreased by 72.5% in plasma and by 63.1% in blood cells relative to SHC value in this time point.

Differences were also observed in the activity of the mitochondrial form of SOD. In blood cells one-day reperfusion did not change the level of Mn-SOD activity. On the contrary, there was a significant reduction of Mn-SOD activity in plasma of experimental animals by about 65% in comparison with SHC (Fig. 3).

DNA damage of blood lymphocytes

Both SSBs and DSBs of purified lymphocytes were measured by SCGE (Fig. 4). In a sham control, SSBs reached a value of 24.73 ± 0.4607% DNA in tail and were more abundant (by about 13.1%) then DSBs (21.49 ± 0.4312% DNA in tail). One day after the ischemic insult DSBs decreased by about 5.21% compared to control value.

On the contrary, SSBs were significantly elevated (by 9.85%) from baseline, and were also more abundant than DSBs (by 20.14%) after the same time of reperfusion.

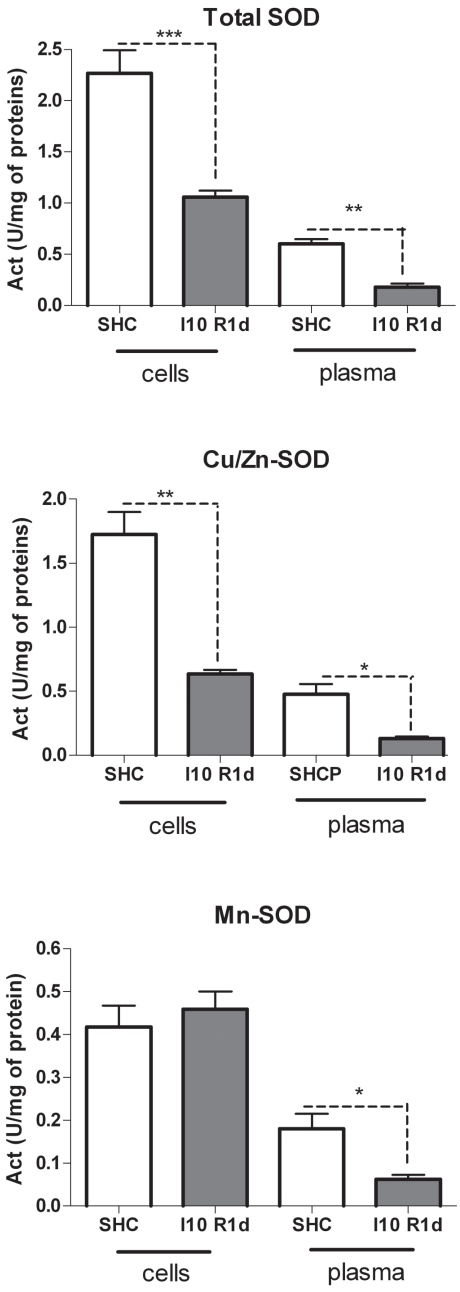

Figure 3. SOD activity in plasma and blood cells one day after transient forebrain ischemia. Data are expressed as mean ± SD. SHC, sham control; I10R1d, global ischemia 10 minutes/recirculation 1 day; Act, SOD activity normalized on the basis of total protein content; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

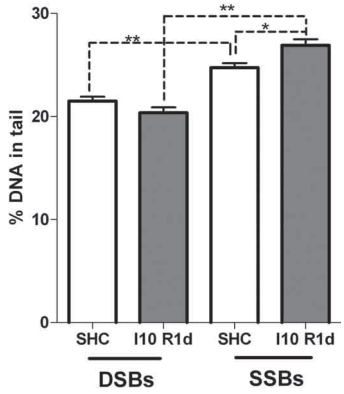

Figure 4. Single (SSBs) and double strand breaks (DSBs) of DNA in peripheral lymphocytes one day after transient forebrain ischemia. Data are expressed as mean ± SD. SHC, sham control; I10R1d, global ischemia 10 minutes/recirculation 1 day; * $p < 0.01$, ** $p < 0.001$.

Discussion

Restoration of blood supply to ischemic tissue leads to a progressive cascade of biochemical and immunological events. Among the mechanisms involved in postischemic injury, excitotoxicity and oxygen free radicals play a central role. Although postischemic recirculation is a significant source of ROS, blood supply restoration seems to be the most important requirement for the survival of affected brain tissue.

In the present study we explored the effect of reperfusion on plasma and blood cells one day after the ischemic insult, the time interval coinciding with a second rise of perfusion (Pulsinelli et al. 1982). Our work is based on our previous study of early reperfusion period after an ischemic attack. In that study, during first two hours of postischemic reperfusion we observed not only a biphasic elevation of glutamate concentration, SOD activity and lymphocyte DNA damage at 40 and 120 min of postischemic reperfusion, but also an important correlation between those parameters. The most interesting time for assessing of postischemic changes in blood is around 40 min of reperfusion when almost all observed parameters reached the highest level (DNA damage is elevated at least about 41%, glutamic acid concentration about 23% and activity of total and Cu/Zn-SOD enzymes is 14.8-fold higher) (Kravcukova et al. 2009). Early stage of recirculation is characterized by postischemic reactive hyperemia followed approx. after 15 to 120 min by hypoperfusion in dependence on brain ischemia duration (Singh et al. 1992). At this time the concentration of extracellular glutamate increases at least ten times, but after blood supply restoration the level of glutamate returns to baseline in 5–20 min. There are scattered reports of delayed two-fold increases in glutamate approx. 3 h after a global insult (Andine et al. 1991). Importance of those reports lies in a possibility of repeated glutamate-induced oxidative stress.

Elevated release of glutamate into extracellular space (Han et al. 2008) and subsequently into blood circulation (Gottlieb

et al. 2003; Teichberg et al. 2009) is an important source of ROS in postischemic organism. Through a breakdown of the glutamate/cystine antiporter concomitant reduction in glutathione synthesis, glutamate affects elevation of intracellular ROS in neurons (Ha and Park 2006) and peripheral lymphocytes (Tuneva et al. 2003; Mashkina et al. 2007).

In prevention of oxidative damage of cells and neutralization of ROS cellular antioxidant systems and different repairing enzymes are involved. Recent studies have provided evidence that under pathologic conditions such as cerebral ischemia, the oxidant-antioxidant balance in brain tissue (Danielisova et al. 2005) as well as in blood is disrupted (Luneva et al. 2005; Kim and Chung 2007).

One of the major antioxidant enzymes is SOD. Brain ischemia and reperfusion results in elevated enzymatic activity of SOD in different brain regions (Danielisova et al. 2005; 2009) as well as in blood plasma (Akcil et al. 2000). Reply of the circulatory systems seems to be pliant with regard to duration and/or method of brain ischemia. In the same model of brain ischemia, blood supply renewing leads to a significant increase in SOD activity after the strong ischemic attack (Luneva et al. 2005) or decrease in SOD activity in plasma and blood cells after the weak ischemia in the same time of early postischemic reperfusion (Ozerol et al. 2009). One-day long postischemic reperfusion in our experiment resulted in suppressed SOD antioxidant capacity. Total SOD and Cu/Zn-SOD activity in blood cells decreased at least by half, and their plasma activity fell as low as to 28% of the preischemic level. In spite of this, the activity of mitochondrial SOD in blood cells stayed unchanged, even while decreasing by 65% in plasma.

Reduced antioxidant activity was observed in patients after ischemic stroke. The SOD activity in the serum of patients with acute cerebral ischemia was significant decrease within 24 h after stroke compared with age-matched control subjects (Spranger et al. 1997). Moreover, it has been shown that Mn-SOD is efficient in protecting against cellular injuries by transient hypoxic conditions (Hirai et al. 2004) and overexpression of Mn-SOD reduced the levels of intracellular ROS and prevented cell death (Keller et al. 1998; Majima et al. 1998; Macmillan-Crow and Cruthirds 2001).

DNA molecule is a target of ROS. Oxidative stress can induce diverse lesions in the DNA, including abasic sites, DNA strand breaks and oxidized bases (Collins and Horvathova 2001). Therefore, DNA SSBs are one of the most frequent DNA lesions in mammalian cells even under physiological conditions.

In several animal studies postischemic reperfusion was found to affect brain DNA and to result in double and single-strand breaks (Chen et al. 1997; Huang et al. 2000; Liu et al. 2007). In circulatory system, 15 min forebrain ischemia leads to increased DNA damage of peripheral lymphocytes, lasting for up to 3 h of reperfusion and decreasing 3 days after ischemic insult (Sivonova et al. 2008). Our investigation of one-day reperfusion after forebrain ischemia shows changes of lymphocyte DNA integrity. We observed mainly a significant increase in SSBs consistent with ROS formation during postischemic reperfusion.

In concert to this, unchanged blood cell Mn-SOD activity one day after the ischemic attack could be explained by postischemic blood cells renewing, for example by stem/progenitor cells as it was observed after stroke related stress (Machalinski et al. 2006).

The present results confirm that one day of recovery after ischemic attack did not return several parameters of the peripheral system to physiological conditions. We can conclude that one day after global cerebral ischemia antioxidant capacity of circulatory system was markedly reduced. We observed an elevated level of glutamic acid in total blood, a drop of total and Cu/Zn-SOD antioxidant activity in plasma and blood cells and, finally, unchanged mitochondrial SOD activity in cellular elements. Damage of peripheral blood lymphocytes showed a significantly elevated level of single strand, but a slightly reduced level of DSBs of the DNA molecule, which may be explained by increased oxidative stress and reduced antioxidant capacity of postischemic organism. This situation could be the reason for immune cell damage and instability and, with regard to the importance of the immune system in postischemic recovery, it could be related to brain tissue damage. Moreover, these circulatory system parameters could be an effective indicator of central nervous system injury.

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