Time Course of Ischemia/Reperfusion-Induced Oxidative Modification of Neural Proteins in Rat Forebrain

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Abstract. Time course of oxidative modification of forebrain neural proteins was investigated in the rat model of global and partial cerebral ischemia/reperfusion. Animals were subjected to 4-vessel occlusion for 15 min (global ischemia). After the end of ischemia and at different reperfusion times (2, 24 and 48 h), lipoperoxidation-dependent and direct oxidative modification neural protein markers were measured in the forebrain total membrane fraction (tissue homogenate). Ischemia itself causes significant changes only in levels of tryptophan and bityrosine fluorescence when compared to controls. All tested parameters of protein modification altered significantly and were maximal at later reperfusion stage. Content of carbonyl group in re-flow period steadily increased and culminated at 48 h of reperfusion. The highest increase in the fluorescence of bityrosines was detected after 24 h of reperfusion and was statistically significant to both sham operated and ischemic groups. The changes in fluorescence intensity of tryptophan decreased during a reperfusion time dependent manner. Formation of lysine conjugates with lipoperoxidation end-products significantly increased only at later stages of reperfusion. Total forebrain membranes from animals subjected to 3-vessel occlusion model to 15 min (partial ischemia) show no altered content of oxidatively modified proteins compared to controls. Restoration of blood flow for 24 h significantly decreased only fluorescence of aromatic tryptophan. Partial forebrain ischemia/reperfusion resulted in no detectable significant changes in oxidative products formation in extracerebral tissues (liver and kidney) homogenates. Our results suggest that global ischemia/reperfusion initiates both the lipoperoxidation-dependent and direct oxidative modifications of neural proteins. The findings support the view that spatial and temporal injury at later stages of ischemic insult at least partially involves oxidative stress-induced amino acid modification. The results might have important implications for the prospective post-ischemic antioxidant therapy.

Key words: Cerebral ischemia — Protein modification — Rat — Fluorescence

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Introduction

Cerebral ischemia results in a cascade of events leading to a number of important cellular changes that evolve over time and space. These include rapid decreases in ATP, loss of ionic homeostasis, excitotoxicity, dysfunction of mitochondria and endoplasmic reticulum and activation of hydrolytic enzymes (Lipton 1999). The possible role of reactive oxygen (nitrogen) species (RO(N)S) in ischemic cell damage have been extensively studied since the early suggestion of Siesjö (1981). The brain is vulnerable to oxidative damage because of a relative lack of antioxidant enzymes and abundance of both the oxidizable substrates and transition metals. Several groups have shown an increased formation of RO(N)S during ischemia induced by experimental cerebral vessels occlusion especially in affected brain regions, such as striatum (Zini et al. 1992; Phillis and Sen 1993) and hippocampus (Piantadosi and Zhang 1996). The role of oxidative stress becomes much greater in case where cerebral blood flow is restored (Oliver et al. 1990; Sakamoto et al. 1991; Dirnagl et al. 1995; Piantadosi and Zhang 1996; Solenski et al. 1997; Murín et al. 2001) because re-flow to previous ischemic brain results in an increase in oxygen level. However, reperfusion period is necessary to salvage the compromised ischemic tissue.

The cellular targets and the mechanisms underlying free radical mediated damage, however, are not yet clear. RO(N)S might affect the structures of both membrane and cytosolic lipids, proteins and other macromolecules including nucleic acids. As result of interactions with unsaturated fatty acids, RO(N)S markedly reduce membrane fluidity by formation of several reactive products such as peroxides, hydroperoxides, aldehydes (Ikeda and Long 1990) and conjugated dienes (Chen and Yu 1994; Kaplan et al. 1995, 2000). It is important to note, that, unlike free radicals, aldehydes generated by RO(N)S-related mechanisms are rather long lived and therefore are able to diffuse from the site of their origin (i.e. membranes) and attack intra- or extracellular targets, which are distant from the initial free radical event.

Another target of RO(N)S is neural proteinous amino acyl side chain structure. Its oxidation forms products such as carbonyl groups (Freeman and Crapo 1982; Stadtman and Oliver 1991), disulfides (Freeman and Crapo 1982), and bityrosines (biTyr), or leads to the modification of tryptophan (Trp) and lipoperoxide-dependent lysine (Lys) adducts (Davies et al. 1987a), all used as markers of oxidative damage to protein. Oxidized proteins show increased susceptibility to proteolytic enzymes, they possess changed electric charge and capability to provoke aggregation or fragmentation (Davies et al. 1987b). As a consequence, mechanisms responsible for both the inhibition of removal mechanisms and consequent accumulation of damaged proteins might be induced.

Several lines of evidence indicate that oxidative stress contributes to delayed neuronal death after global ischemia, suggesting that RO(N)S formation may cooperate in series of molecular events that link ischemic injury to neuronal death. Many important ion transporting enzymes are functionally inhibited following even quite short exposures with RO(N)S *in vitro* including plasmalemmal Na^+ pump

(Elmoselhi et al. 1994) and both types of Ca^{2+} pumps (Racay et al. 1994, 1998; Zaidi and Michaelis 1999; Lehotsky et al. 2002a). Oxidation by free radicals changes enzyme activity (Matejovicova et al. 1996; Lehotsky et al. 1999, 2002b) and makes it susceptible to calpain-mediated proteolysis (Zolotarjova et al. 1994). In addition, oxidative damage to endoplasmic reticulum is believed to be implicated in ischemic cell death (Hayashi et al. 2003; Lehotský et al. 2003).

Thus, elucidation of the extent and the role of oxidative stress in the brain after ischemia/reperfusion is of great importance. A better understanding of a timing and factors that infuence RO(N)S formation is required for effective antioxidant intervention. Although, RO(N)S have been postulated to play an important role in the progression of reperfusion injury, the time course of oxidative damage is still poorly characterized. In our previous paper we have described formation of lipoperoxidation products induced by transient global ischemia/reperfusion injury in total membranes (tissue homogenate) (Murín et al. 2001). In the present study we analyzed the time course formation of lipoperoxidation-dependent and direct oxidative products of proteins in total forebrain membranes (tissue homogenate) following global ischemia/reperfusion and partial forebrain ischemia/reperfusion in rats.

Materials and Methods

Animals

The experimental studies used Wistar rats (breeding station of the Slovak Academy of Sciences, Dobrá Voda, Slovakia) aged 4 months (weighing 370 ± 15 g). Animals were randomly assigned to one of three treatment groups and were identified only by a serial code number to blind investigators to the treatment group. The treatment groups consisted of a control (sham-operated) group, an ischemia group and ischemia/reperfusion group. The animals were allowed free access to food (commercial laboratory chow) and water; they were exposed to a light/dark cycle (12 : 12 h), housed five *per* cage in air-conditioned room.

Induction of 4-vessel global ischemia

A 4-vessel occlusion procedure was used to induce global cerebral ischemia (Pulsinelli at al. 1983). On day 1, the both vertebral arteries were cauterized under halothane anesthesia. The carotid arteries were exposed and loops of silastic tubing were placed loosely around them to allow rapid subsequent exposure of these vessels. On day 2, the carotid arteries were located again and were occluded with artery clamps for 15 min. Ischemia were reversed after 15 min by removing the carotid artery clamps following by 2, 24 and 48 h of reperfusion. Body temperature was monitored with a rectal probe during ischemia. If necessary, temperature was maintained with a heating lamp.

Following the given recirculation period, rats were sacrificed by decapitation. The brains were first washed in ice-cold homogenizing solution containing 0.32

mol/l sucrose, 10 mmol/l HEPES with pH 7.4. The forebrain without cerebellum was dissected at 4 °C and homogenized in Potter–Elvehjem homogenizer in ratio 1 g tissue in 10 ml homogenizing solution. Homogenate was centrifuged (1200 × *g* for 10 min) to remove the debris (total membrane fraction). Protein concentration was determined by the Lowry method. Control group was sham-operated and total membrane fraction (homogenate) was prepared as described above.

Induction of 3-vessel partial ischemia

A 3-vessel occlusion model was used to induce partial cerebral ischemia (Brint et al. 1988). Ischemia was induced as described above instead of that on day 1 only the left vertebral artery was cauterized. Ischemia was reversed after 15 min by removing the clamps from both carotid arteries following by 24 h of reperfusion. On the day of the experiments, animals were killed by decapitation and brain, liver and kidney were immediately removed. The olfactory bulbs and pons/medulla were discarded. Forebrain without cerebellum, kidneys and liver were weighed and kept chilled until homogenization. Control group was sham-operated and total membrane fractions (homogenates) were prepared as described above and were kept at −70 °C until used for determinations.

To avoid production of free radicals which are potentially induced by isolation and by tissues processing, the ice-cold homogenization buffer was used and all samples were handled and processed in an identical fashion.

Carbonyl groups measurement

Content of carbonyl groups were determined as a product of protein reaction with 2,4-diphenyl hydrasine and assessed by spectrophotometry at 365 nm after precipitation with trichloroacetic acid and solubilization in guanidium chloride (Levine et al. 1994).

Free sulfhydryl groups measurement

Contents of free sulfhydryl (SH) groups in forebrain homogenate were measured by 2,2-dithibisnitrobenzoic assay (Hu 1994) in Tris buffer, pH 8.2; absorbance was read at 412 nm.

Fluorescence measurements

Fluorescence measurements were performed in solution containing 50 μg of homogenate protein *per* ml, 10 mmol/l HEPES, 100 mmol/l KCl, pH 7.0 at 25 °C on a Shimadzu spectrofluorophotometer RF-540 (Japan).

Fluorescence emission spectra (300 to 450 nm, slit width 2 nm) of Trp were measured with excitation at 295 nm (slit width 1 nm).

Fluorescence emission spectra of biTyr, product of tyrosine oxidation, were recorded in range 380 to 440 nm, (slit width 2 nm) at excitation wavelength 325 nm (slith width 2 nm) (Giulivi and Davies 1994).

Emission spectra of Lys conjugates with lipid peroxidation products (425 to 480 nm, slit width 2 nm) were recorded at 365 nm (slit width 2 nm) and excitation

spectra of conjugates of Lys (325 to 380 nm, slit width 2 nm) were measured with emission at 440 nm (slit width 2 nm) (Dousset et al. 1994).

Protein determination

Protein was measured by the method of Lowry et al. (1951) using bovine serum albumine as standard.

Data analysis

The results were presented as mean ± S.E.M. One-way analysis of variance with post-hoc comparisons by Student–Neuman–Keuls test was carried out to test for differences among groups. A value of $p < 0.05$ was considered to be statistically significant.

Results

Global transient ischemia/reperfusion

In our previous paper we have shown that transient global forebrain ischemia for 15 min induces abrupt lipid peroxidation as measured by the formation of conjugated dienes and levels of thiobarbituric acid reactive substances (TBARS) in total membranes (tissue homogenate) (Murín et al. 2001). In addition, the formation of lipoperoxidation products increased as early as 2 h of recirculation and reached the maximal values at later blood re-flow period. The differences were statistically significant when compared to both the sham-operated and the ischemic tissue.

In order to qualify whether ischemia-induced oxidative stress and/or lipid peroxidation lead also to structural modifications of cellular proteins we measured in total membrane fraction (tissue homogenate) oxidative products and fluorescence of several amino acids as indicators of oxidative damage to proteins. To assess their

Table 1. Level of free SH groups and fluorescence intensity of tryptophan (Trp) in rat forebrain homogenate after ischemia (ISCH) and various duration (2, 24 and 48 h) of reperfusion (REP) (4-vessel occlusion model, 4 months)

	free SH groups mmol/mg of protein	fluorescence intensity of Trp (a.u.)
control	0.347 ± 0.038	75.6 ± 0.5
15-min ISCH	0.345 ± 0.029	71.1 ± 0.9***
2-h REP	0.226 ± 0.013*+	70.4 ± 0.5***
24-h REP	0.169 ± 0.018 ***+++	68.0 ± 0.7***+
48-h REP	0.201 ± 0.012**++	60.9 ± 1.1***+++

Values represent means ± S.E.M. of 7–12 experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; significantly different as compared to control. + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$; significantly different as compared to ISCH (adapted from Murn et al. 2001).

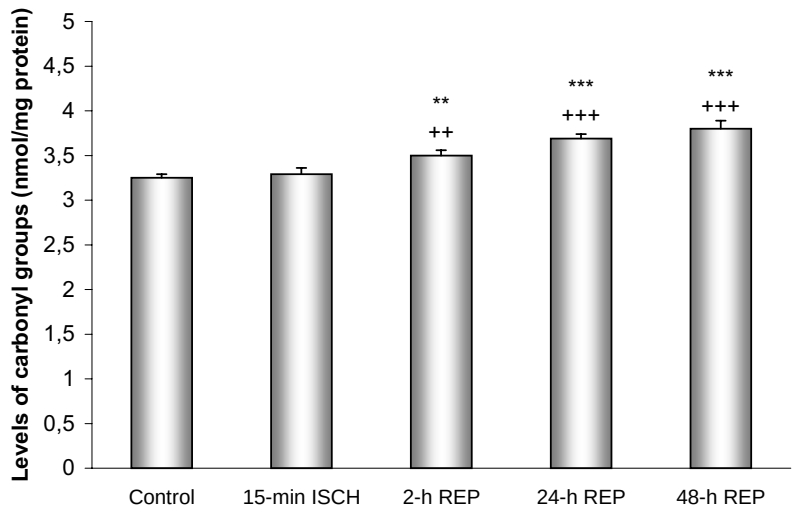

Figure 1. Levels of carbonyl groups in rat forebrain homogenate after 15 min of ischemia (ISCH) and 2, 24 and 48 h of reperfusion (REP) (4-vessel occlusion model, 4 months). Values represent means ± S.E.M. of 7–12 experiments. ** $p < 0.01$, *** $p < 0.001$; significantly different as compared to control. ++ $p < 0.01$, +++ $p < 0.001$; significantly different as compared to ischemia.

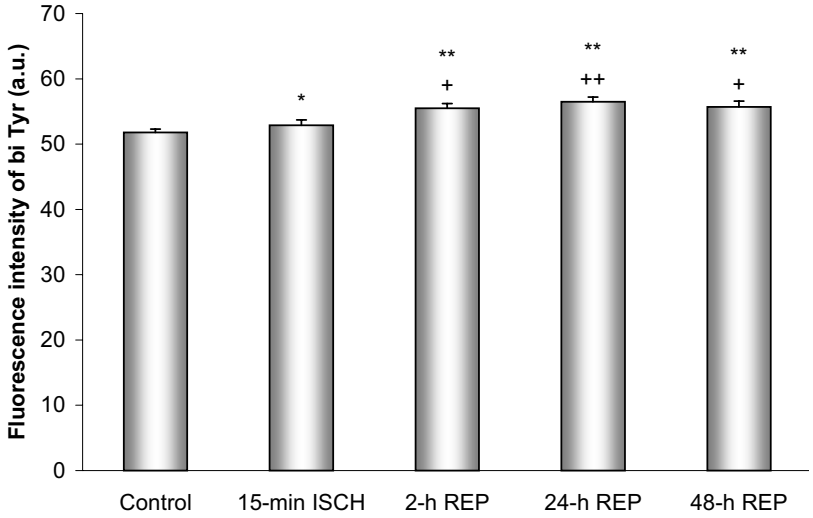

Figure 2. Effect of 15 min of ischemia (ISCH) and various duration (2, 24 and 48 h) of reperfusion (REP) on fluorescence intensity of bityrosine (biTyr) in rat forebrain homogenate (4-vessel occlusion model, 4 months). The results are expressed as means ± S.E.M. of 7–12 experiments. * $p < 0.05$, ** $p < 0.01$; significantly different as compared to control. + $p < 0.05$, ++ $p < 0.01$; significantly different as compared to ischemia. Fluorescence intensity of biTyr is represented in arbitrary units (a.u.).

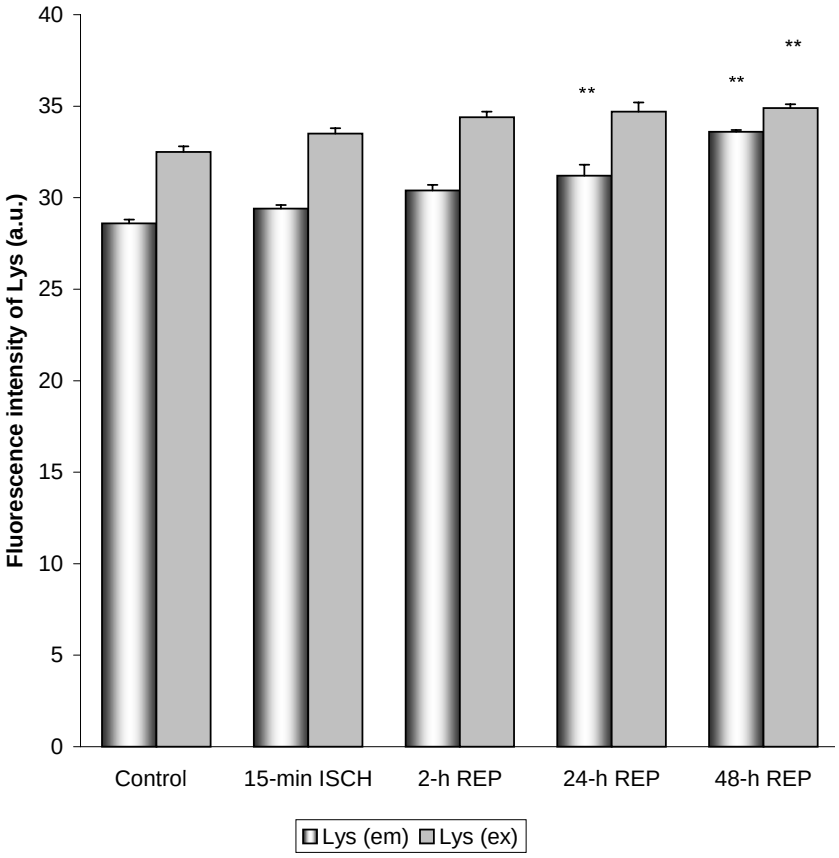

Figure 3. Effect of 15 min of ischemia (ISCH) and various duration (2, 24 and 48 h) of reperfusion (REP) on fluorescence intensity of lysine (Lys) conjugates, Lys emission at 365 nm – Lys (em), Lys excitation at 440 nm – Lys (ex) in rat forebrain homogenate (4-vessel occlusion model, 4 months). The results are expressed as means ± S.E.M. of 7–12 experiments. ** $p < 0.01$, *** $p < 0.001$; significantly different as compared to control. Fluorescence intensity of conjugates of Lys (em), Lys (ex) is represented in arbitrary units (a.u.).

structural modifications we determined: i) content of total SH groups, ii) level of intrinsic fluorescence of aromatic amino acid Trp (Tab. 1), iii) amount of carbonyl groups introduced in the protein molecules (Fig. 1), iv) fluorescence of biTyr as a measure of cross-linking of intra- and extra-cellular proteins (Fig. 2) and v) Lys conjugates with lipid peroxidation end-products (Fig. 3).

As we have shown in previous paper (Murín et al. 2001), even single global ischemia for 15 min duration causes an abrupt formation of lipoperoxidation products. In this work, from all tested parameters of oxidative damage to proteins, is-

chemia itself causes significant changes only in levels of Trp and biTyr fluorescence when compared to levels detected in sham-operated animals (Tab. 1, Fig. 2). Moreover, as early as recirculation starts, all tested parameters of protein modification significantly altered and mostly reached maximal alterations at later reperfusion period (48 h). Content of carbonyl groups (Fig. 1) in re-flow period steadily increased, culminated at 48 h of reperfusion with high statistical significance between control ($p < 0.001$) and ischemia ($p < 0.001$).

The highest increase in the fluorescence of biTyr (Fig. 2) was detected after 24 h of reperfusion and was statistically significant to both sham-operated ($p < 0.01$) and ischemic groups ($p < 0.05$). The changes in fluorescence intensity of Trp decreased in a reperfusion time dependent manner.

To gain further insight into the ischemia-induced lipid peroxidation protein modification we measured excitation and emission fluorescence spectra corresponding to Lys residues derivatized by lipid peroxidation end-products. In spite of very early lipoperoxidation products formation after ischemia (Murín et al. 2001), Fig. 3 shows that ischemia followed blood re-flow induces significant increase in aminoacid conjugates (excitation and emission spectra) only at later stages of reperfusion ($7.4 \pm 0.6\,\%$ after 48 h when compared to control, $p < 0.05$).

Partial/focal ischemia/reperfusion

We compared extent of ischemia/reperfusion-induced liporeroxidation and protein oxidation products formation by transient (15 min) partial ischemia and ischemia followed by restoration of blood flow for 24 h in total membrane fraction (homogenate) from potentially affected forebrain area. Transient ischemia/reperfusion induces formation of lipoperoxidation, as detected by TBARS (results not shown).

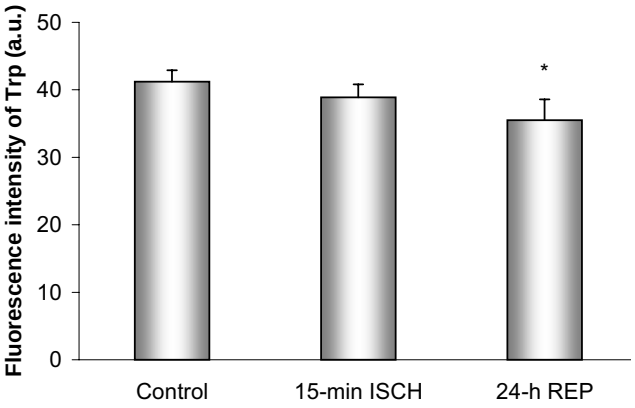

Figure 4. Effect of 15 min of ischemia (ISCH) and 24 h of reperfusion (REP) on fluorescence intensity of tryptophan (Trp) in rat forebrain homogenate (3-vessel occlusion model, 4 months). Values represent means ± S.E.M. of 6 determinations. * $p < 0.05$. Fluorescence intensity of Trp is represented in arbitrary units (a.u.).

However, in our experimental settings, of all parameters of protein oxidation, ischemia itself did not affect formation of any oxidative products compared to sham-operated controls. Restoration of blood flow for 24 h after ischemia significantly decreased only fluorescence of Trp in comparison with controls and ischemic level (Fig. 4). There were no significant changes in the level of conjugated dienes and fluorescence of biTyr and Lys derivatives.

Oxidative stress initiated during ischemia/reperfusion of the brain may also have a potentially deleterios effect on extra-cerebral tissues. In this experimental setting, ischemia followed blood re-flow resulted in no detectable significant changes in oxidative products formation in liver and kidney homogenates (results not shown).

Discussion

Global transient ischemia induced by 4-vessel occlusion in rats is a model for human cerebral ischemia resulting from transient cardiac arrest (Lipton 1999). 3-vessel occlusion in rats is a model of partial/focal ischemia of cerebral tissue. There is an increasing evidence that the brain damage produced by cerebral ischemia develops over a longer period of time. Oxidative stress is well recognized factor of ischemic/reperfusion injury. Experiments show that in one line of acute RO(N)S effect, reactive species can trigger delayed neuronal death even after apparent metabolic recovery (Dalton et al. 1999). Evidence suggests that lipid peroxidation plays a widespread role in neuronal cell death. Lipoperoxidation results among others in loss of membrane integrity, impairement of the function of membrane transport proteins and channels and parallels with selective post-ischemic vulnerability of the brain (Hall et al. 1993; Racay et al. 1996; Mattson 1998). Our previous results in both models of cerebral ischemia in rats have shown sustained elevation of markers of lipid peroxidation, which was maximal at later period of blood re-flow (Murín et al. 2001). As also shown in gerbils, the increased level of lipid peroxidation measured as malonedialdehyde reactive substances, or as 4-hydroxy, 2-nonenal immunoreactivity persists over several days after brief global forebrain ischemia (Caldwell et al. 1995; Urabe et al. 2000) and is also significantly increased in human hippocampus after cardio-respiratory arrest (global ischemia) (McKracken et al. 2001). It is interesting to note that the time course of increased lipoperoxidation is simultaneous with that of post-ischemic neuronal degeneration (Hall et al. 1993). Nevertheless, the mechanisms underlying the oxidative damage at later stages after initial ischemic insult are not completely understood. Lipoperoxidation may secondary affect membrane proteins *via* modifications of their structure and thus may further deteriorate ischemia-induced neural damage. The causative evidence for the ischemia-induced oxidative damage to proteins and loss of functional properties of neuronal proteins has been suggested only during last few years (Lipton 1999).

In order to qualify whether ischemia-induced oxidative stress/lipid peroxidation leads also to structural modifications of cellular proteins, we have measured

fluorescence of several amino acids as indicators of oxidative damage to proteins. In our previous work, we have shown that global ischemia induces significant decrease in the total SH groups content and decrease in fluorescence of aromatic amino acid Trp. In this study, we documented that in addition to previous results, ischemia followed by reperfusion induces structural changes in the cellular proteins also in another amino acids as detected by the increase in the content of carbonyl groups, biTyr fluorescence and fluorescence of Lys adducts. The damage has apparent time dependence on the course of reperfusion and partially delayes lipoperoxidation time profile. From the experiments performed *in vitro*, it is known that effect of free radicals affect both proteinous and lipidic part of the membranes, however, radicals strongly inhibit important enzymes regulating neuronal ion homeostasis (Matejovicova et al. 1996; Lehotsky et al. 1999). The modified amino acids may have critical role in the function of several important neural proteins such as ion transport proteins (Breier and Ziegelhoffer 2000; Lehotsky et al. 2002a,b; Kaplán et al. 2003), glutamate transporter (Rao et al. 2000) and endoplasmic proteins involved in the reticular stress response to ischemia (Hayashi et al. 2003). As a consequence, damaged proteinous products such as of intra- and extramolecular dityrosine cross-links and protein adducts with lipoperoxidation products can be degraded or accumulated in the cells. In addition, altered redox balance may potentially change activation or silencing of genes encoding transcription factors such as AP-1, NF-κB, antioxidant defense enzymes, and structural proteins which may modulate signal transduction cascades and redirect gene expression in neural cells (Lipton 1999; Liu et al. 2003).

Physiologically, the rates of free radical production and elimination are equal, leading to a steady state that is presumably tolerated by the cell. Ischemia creates several conditions that could account for the increased net production of free radicals (Freeman and Crapo 1982; Ikeda and Long 1990) or an impairment of cellular defenses that normally protect against such damage (Sims et al. 1998). The massive production of free radicals during ischemia/reperfusion was observed in plenty of studies by indirect spin trapping measurements and chemiluminiscence (Oliver et al. 1990; Sakamoto et al. 1991; Zini et al. 1992; Phillis and Sen 1993; Dirnagl et al. 1995; Piantadosi and Zhang 1996; Solenski et al. 1997). The most robust and significant alteration in the antioxidant defence is a decrease in glutathione content (Schulz et al. 2000). In an attempt to elucidate the factors that might influence free radical formation after ischemia/reperfusion, Candelario-Jalil et al. (2001) have found the reduced antioxidant defence capacity of both glutathione peroxidase and glutathione reductase in gerbils in reperfusion period in parallel with sustained elevation of manganese superoxidismutase activity. Recent experiments also shown that both cyclooxygenase (COX) isoforms are involved in the oxidative damage induced by global ischemia in gerbils but only COX-2 contributes to brain injury in focal ischemia in rats (Candelario-Jalil et al. 2003). Other mechanisms which might account for the late increase in oxidative damage is probably the delayed induction of RO(N)S generating enzyme like nitric oxide synthase, heme oxygenase and phospholipase A2 (Pavel et al. 2001; Adibhatla and Hatcher 2003). Prevention

of increased free radicals production by antioxidants had shown at least partial protective effect on the post-ischemic neuronal injury (Lipton 1999; Stolc 1999; Candelario-Jalil et al. 2003).

The presented findings demonstrate that accumulation of oxidatively modified amino acyl residues can be observed as soon as after 15 min of global ischemia with a maximal level of later period of blood re-flow. It suggests that forebrain neural cells are highly susceptible to oxidative changes induced by brief global ischemic episode appeared mainly in later reperfusion period, which is very similar to the time course of pattern of neuronal vulnerability assessed histopathologically (Kirino 1982; Nitatori et al. 1995). The histochemical changes induced by global ischemia in rats has also been shown to parallel iron deposition in hippocampal CA1 neurons in 4-vessel occlusion model of global ischemia (Danielisova et al. 2002). Since iron is an important activator of free radicals formation, elevated deposition of iron may also lead to consequent post-ischemic oxidative neuronal dysfunction. The time course of oxidative products formation documented in our study is also in one line with the work of Candelario-Jalil et al. (2001), which documents disturbance in oxidant-antioxidant balance at later stages after the ischemic insult on gerbils. Thus, in our study, oxidative alterations of neural proteins seen after ischemia/reperfusion may at least partially explain functional postischemic disturbances of neuronal ion transport mechanisms (Lehotsky et al. 2002b) and ischemia-induced inhibition of global proteosynthesis (Burda et al. 2003; Hayashi et al. 2003), which both are implicated in neuronal cell damage and/or recovery from ischemic insult (Račay and Lehotský 1996; Lipton 1999).

3-vessel occlusion model of partial ischemia/reperfusion injury in the rat has gained increasing acceptance owing to its relevance to human clinical settings (Brint et al. 1988). While this model is clinically relevant, the effect of vessel occlusion on energy metabolism is more difficult to interpret because of the multiple regions of varying blood flow and zones of partially ischemic tissue (Welsh 1984). Experimental evidence from this model of ischemia indicates that lipoperoxidation may also contribute to damage to neurons in affected brain areas and the early indirect measurements of TBA reactants accumulation strongly indicated that free radicals were produced within 30 min of initiating ischemia (for review see Lipton 1999). In spite of that, in our experimental setting we were not able to detect any statistically significant alterations of protein oxidation parameters by ischemia itself and restoration of blood flow for 24 h after ischemia significantly decreased only fluorescence of aromatic amino acid Trp, which is considered as moderately specific marker of oxidative damage. There were no significant changes in the level of conjugated dienes and fluorescence more selective marker such as of biTyr and Lys derivatives. Clearly, the possibility of variable infarct volume in the vessels territory from rat to rat which can results in a relative high standard deviation must be considered to explain our findings (Karpiak et al. 1989). Another explanation could be that the percentage of oxidatively vulnerable cells, which is present in tissue used for total membrane preparation, is too low to detect oxidative changes (Brint et al. 1988). The results also raise another question of whether oxidation of

proteins in this model starts at quantitatively lower levels in the relatively early reperfusion period (Solenski et al. 1997). Additional experiments are needed to clarify those questions by analyzing the selectively vulnerable brain areas affected by partial ischemia.

In conclusion, our results suggest that global ischemia/reperfusion initiates both the lipoperoxidation-dependent and direct oxidative modifications of neural proteins. The findings support the view that spatial and temporal injury at later stages of ischemic insult involves at least partially oxidative stress-induced amino acid modification. In addition, the combination of amino acid modification, rather than oxidation of amino acids of one kind is resposible for postischemic oxidative injury. These results and our previous laboratory findings described in Murín et al. (2001) might have important implications for the prospective post-ischemic antioxidant therapy in global ischemia due to the maximal appearance of oxidative products in the late reperfusion stages.

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References

Adibhatla R. M., Hatcher J. F. (2003): Citicoline decreases phospholipase A2 stimulation and hydroxyl radical generation in transient cerebral ischemia. J. Neurosci. Res. **73**, 308—315

Breier A., Ziegelhöffer A. (2000): "Lysine is the Lord", thought some scientists in regard to the group interacting with fluorescein isothiocyanate in ATP-binding sites of P-type ATPase. But, is it not cysteine? Gen. Physiol. Biophys. **19**, 253—263

Brint S., Jacewicz M., Kiessling M., Tanabe J., Pulsinelli W. (1988): Focal brain ischemia in the rat: methods for reproducible neocortical infarction using tandem occlusion of the distal middle cerebral and ipsilateral common carotid arteries. J. Cereb. Blood Flow Metab. **8**, 474—485

Burda J., Hrehorovska M., Bonilla L. G., Danielisova V., Cizkova D., Burda R., Nemethova M., Fando J. L., Salinas M. (2003): Role of protein synthesis in the ischemic tolerance acquisition induced by transient forebrain ischemia in the rat. Neurochem. Res. **28**, 1213—1219

Caldwell M., O'Neill M., Early B., Kelly J. P., Leonard B. E. (1995): NG-nitro-L-arginine methyl ester protects against lipid peroxidation in the gerbil following cerebral ischaemia. Eur. J. Pharmacol. **285**, 203—206

Candelario-Jalil E., Mhadu N. H., Al-Dalain S. M., Martinez G., Leon O. S. (2001): Time course of oxidative damage in different brain regions following transient cerebral ischemia in gerbils. Neurosci. Res. **41**, 233—241

Candelario-Jalil E., Gonzalez-Falcon A., Garcia-Cabrera M., Alvarez D., Al-Dalain S., Martinez G., Leon O. S., Springer J. E. (2003): Assessment of the relative contribution of COX-1 and COX-2 isoforms to ischemia-induced oxidative damage and neurodegeneration following transient global cerebral ischemia. J. Neurochem. **86**, 545—555

Dalton T. P., Shertzer H. G., Puga A. (1999): Regulation of gene expression by reactive oxygen. Annu. Rev. Pharmacol. Toxicol, **39**, 67—101

Danielisova V., Gottlieb M., Burda J. (2002): Iron deposition after transient forebrain ischemia in rat brain. Neurochem. Res. **27**, 237—242

Davies K. J., Delsignore M. E., Lin S. W. (1987a): Protein damage and degradation by oxygen radicals. II. Modification of amino acids. J. Biol. Chem. **262**, 9902—9907

Davies K. J., Lin S. W., Pacifici R. E. (1987b): Protein damage and degradation by oxygen radicals. IV. Degradation of denatured protein. J. Biol. Chem. **262**, 9914—9920

Dirnagl U., Lindauer U., Them A., Schreiber S., Pfister H. W., Koedel U., Reszka R., Freyer D., Villringer A. (1995): Global cerebral ischemia in the rat: online monitoring of oxygen free radical production using chemiluminescence *in vivo*. J. Cereb. Blood Flow Metab. **15**, 929—940

Dousset N., Ferretti G., Taus M., Valdiguie P., Curatola G. (1994): Fluorescence analysis of lipoprotein peroxidation. Methods Enzymol. **233**, 459—469

Elmoselhi A., Butcher A., Samson S. E., Grover A. K. (1994): Coronary artery contractility, Na^+-pump and oxygen radicals. Gen. Physiol. Biophys. **13**, 247—256

Freeman B. A., Crapo J. D. (1982): Biology of disease: free radicals and tissue injury. Lab. Invest. **47**, 412—426

Giulivi C, Davies K. J. (1994): Dityrosine: a marker for oxidatively modified proteins and selective proteolysis. Methods Enzymol. **233**, 363—371

Hall E. D., Andrus P. K., Althaus J. S., VonVoighlander P. F. (1993): Hydroxyl radicals production and lipid peroxidation parallels selective post-ischemic vulnerability in gerbil brain. J. Neurosci. Res. **34**, 107—112

Hayashi T., Saito A., Okuno S., Ferrand-Drake M., Dodd R. L., Nishi T., Maier C. M., Kinouchi H., Chan P. H. (2003): Oxidative damage to the endoplasmic reticulum is implicated in ischemic neuronal cell death. J. Cerebr. Blood Flow Metab. **23**, 1117—1128

Hu M. L. (1994): Measurement of protein thiol groups and glutathione in plasma. Methods Enzymol. **233**, 380—385

Chen J. J., Yu B. P. (1994): Alterations in mitochondrial membrane fluidity by lipid peroxidation products. Free Radic. Biol. Med. **17**, 411—418

Ikeda Y., Long D. M. (1990): The molecular basis of brain injury and brain edema: the role of oxygen free radicals. Neurosurgery **27**, 1—11

Kaplan P., Racay P., Lehotsky J., Mezesova V. (1995): Change in fluidity of brain endoplasmic reticulum membranes by oxygen free radicals: a protective effect of stobadine, alpha-tocopherol acetate, and butylated hydroxytoluene. Neurochem. Res. **20**, 815—820

Kaplan P., Doval M., Majerova Z., Lehotsky J., Racay P. (2000): Iron-induced lipid peroxidation and protein modification in endoplasmic reticulum membranes. Protection by stobadine. Int. J. Biochem. Cell. Biol. **32**, 539—547

Kaplan, P., Babusikova E., Lehotsky J., Dobrota D. (2003): Free radicals induced protein modification and inhibition of Ca-ATPase of cardiac sarcoplasmic reticulum. Mol. Cell. Biochem. **248**, 41—47

Karpiak S. E., Taglavia A., Wakade C. G. (1989): Animal models for the study of drugs in ischemic stroke. Annu. Rev. Pharmacol. Toxicol. **29**, 403—414

Kirino T. (1982): Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res. **239**, 57—69

Lehotsky J., Kaplan P., Racay P., Matejovicova M., Drgova A., Mezesova V. (1999): Membrane ion transport systems during oxidative stress in rodent brain: protective effect of stobadine and other antioxidants. Life Sci. **65**, 1951—1958

Lehotský J., Kaplán P., Murín R., Raeymaekers L. (2002a): The role of plasma membrane Ca^{2+} pumps (PMCAs) in pathologies of mammalian cells. Front. Biosci. **7**, d53—84

Lehotský J., Kaplán P., Matejovičová M., Murín R., Račay P., Raeymaekers L. (2002b): Ion transport systems as targets of free radicals during ischemia/reperfusion injury. Gen. Physiol. Biophys. **21**, 31—37

Lehotský J., Kaplán P., Babušíková E., Strapková A., Murín R. (2003): Molecular pathways of endoplasmic reticulum dysfunctions: Possible cause of cell death in the nervous system. Physiol. Res. **52**, 269—273

Levine R. L., Williams J. A., Stadtman E. R., Shacter E. (1994): Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol. **233**, 346—357

Lipton P. (1999): Ischemic cell death in brain neurons. Physiol. Rev. **79**, 1431—1568

Liu P. K. (2003): Ischemia-reperfusion-related repair deficit after oxidative stress: implications of faulty transcripts in neuronal sensitivity after brain injury. J. Biomed. Sci. **10**, 4—13

Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J. (1951): Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265—275

Matejovicova M., Machac S., Lehotsky J., Jakus J., Mezesova V. (1996): Synaptosomal Na, K-ATPase during forebrain ischemia in Mongolian gerbils. Mol. Chem. Neuropathol. **29**, 67—78

Mattson M. P. (1998): Modification of ion homeostasis by lipid peroxidation: roles in neuronal degeneration and adaptive plasticity. Trends Neurosci. **20**, 53—57

McKracken E., Graham D. I., Nilsen M., Stewart J., Nicoll J. A., Horsburgh K. (2001): 4-hydroxynonenal immunoreactivity is increased in human hippocampus after global ischemia. Brain Pathol. **11**, 414—421

Murín R., Drgová A., Kaplán P., Dobrota D., Lehotský J. (2001): Ischemia/reperfusion-induced oxidative stress causes structural changes of brain membrane proteins and lipids. Gen. Physiol. Biophys. **20**, 431—438

Nitatori T., Sato N., Waguri S., Karasawa Y., Araki H., Shibanai K., Kominami E., Uchiyama Y. (1995): Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. J. Neurosci. **15**, 1001—1011

Oliver C. N., Starke-Reed P. E., Stadtman E. R., Liu G. J., Carney J. M., Floyd R. A. (1990): Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion-induced injury to gerbil brain. Proc. Natl. Acad. Sci. U.S.A. **87**, 5144—5147

Pavel J., Lukacova N., Marsala J., Marsala M. (2001): The regional changes of the catalytic NOS activity in the spinal cord of the rabbit after repeated sublethal ischemia. Neurochem. Res. **26**, 833—839

Phillis J. W., Sen S. (1993): Oxypurinol attenuates hydroxyl radical production during ischemia/reperfusion injury of the rat cerebral cortex: an ESR study. Brain Res. **628**, 309—312

Piantadosi C. A., Zhang J. (1996): Mitochondrial generation of reactive oxygen species after brain ischemia in the rat. Stroke **27**, 327—331

Pulsinelli W. A., Levy D. E., Duffy T. E. (1983): Cerebral blood flow in the four-vessel occlusion rat model. Stroke **14**, 832—834

Račay P., Lehotský J. (1996): Intracellular and molecular aspects of Ca^{2+}-mediated signal transduction in neuronal cells. Gen. Physiol. Biophys. **15**, 273—289

Racay P., Bezakova G., Kaplan P., Lehotsky J., Mezesova V. (1994): Alteration in rabbit brain endoplasmic reticulum Ca^{2+} transport by free oxygen radicals *in vitro*. Biochem. Biophys. Res. Commun. **199**, 63—69

Račay P., Kaplán P., Lehotský J. (1996): Control of Ca^{2+} homeostasis in neuronal cells. Gen. Physiol. Biophys. **15**, 193—210

Racay P., Qteishat A. W., ElKambergy H. M., Mezesova V., Lehotsky J. (1998): Fe^{2+}-induced inhibition of gerbil forebrain microsomal Ca^{2+}-ATPase: effect of stobadine, glutathione and combination of both antioxidants. Biochim. Biophys. Acta **1370**, 119—126

Rao V. L. R., Rao A. M., Dogan A., Bowen K. K., Hatcher J. F., Rothstein J. D., Dempsey R. J. (2000): Glial glutamate transporter GLT-1 down regulation preceds delayed neurnal death in gerbil hippocampus following transient global ischemia. Neurochem. Int. **36**, 531—537

Sakamoto A., Ohnishi S. T., Ohnishi T., Ogawa R. (1991): Relationship between free radical production and lipid peroxidation during ischemia-reperfusion injury in the rat brain. Brain Res. **554**, 186—192

Schulz J. B., Lindeneu J., Seyfried J., Dichgans J. (2000): Glutathione, oxidative stress and neurodegeneration. Eur. J. Biochem. **267**, 4904—4911

Siesjö B. K. (1981): Cell damage in the brain: a speculative synthesis. J. Cereb. Blood Flow Metab. **1**, 155—185

Sims N. R., Williams V. K., Zaidan E., Powell J. A. (1998): The antioxidant defences of brain mitochondria during short-term forebrain ischemia and recirculation in the rat. Mol. Brain Res. **60**, 141—149

Solenski N. J., Kwan A. L., Yanamoto H., Bennett J. P., Kassell N. F., Lee K. S. (1997): Differential hydroxylation of salicylate in core and penumbra regions during focal reversible cerebral ischemia. Stroke **28**, 2545—2551

Stadtman E. R., Oliver C. N. (1991): Metal-catalyzed oxidation of proteins. Physiological consequences. J. Biol. Chem. **266**, 2005—2008

Stolc S. (1999): Indole derivatives as neuroprotectants. Life Sci. **65**, 1943—1950

Urabe T., Yamasaki Y., Hattori N., Yoshikawa M., Uchida K., Mizuno Y. (2000): Accumulation of 4-hydroxynonenal-modified proteins in hippocampal CA1 pyramidal neurons preceds delayed neuronal damage in the gerbil brain. Neuroscience **100**, 241—150

Welsh F. A. (1984): Regional evaluations of ischemic metabolic interactions. J. Cerebr. Blood Flow Metab. **4**, 309—315

Zaidi A., Michaelis M. L. (1999): Effects of reactive oxygen species on brain synaptic plasma membrane Ca^{2+}-ATPase. Free Radic. Biol. Med. **27**, 810—821

Zini I., Tomasi A., Grimaldi R., Vannini V., Agnati L. F. (1992): Detection of free radicals during brain ischemia and reperfusion by spin trapping and microdialysis. Neurosci. Lett. **138**, 279—282

Zolotarjova N., Ho C., Mellgren R. L., Askari A., Huang W. H. (1994): Different sensitivities of native and oxidized forms of Na^+/K^+-ATPase to intracellular proteinases. Biochim. Biophys. Acta **1192**, 125—131

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