

Changes of Phospholipid Composition and Superoxide Dismutase Activity During Global Brain Ischemia and Reperfusion in Rats

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Abstract. Alterations in phospholipid content and Cu/Zn superoxide dismutase (SOD) activity were examined in rat brain after 15 min of global ischemia (four-vessel occlusion) followed by 2-, 24- or 48-h reperfusion. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE), the main brain phospholipids, were markedly decreased in ischemic rats and remained decreased during the whole reperfusion period. Concentrations of phosphatidylinositol (PI) and sphingomyelin (SM) were also significantly reduced during ischemia but recovered during reperfusion period. In contrast, phosphatidylserine (PS) and lysophospholipids (LysoPL) were unchanged during ischemia but were elevated after 24 h of reperfusion. Significant reductions in blood plasma phospholipids were also demonstrated. 24–48 h of reperfusion markedly decreased PE, PC and PS contents, while the concentrations were almost unchanged by ischemia alone. Brain SOD activity decreased significantly during ischemia and was recovered to control value already after 2 h of reperfusion. These results suggest that ischemia/reperfusion is accompanied by a significant and selective degradation of brain phospholipids that may be attributable to oxidative stress and activation of phospholipases.

Key words: Brain ischemia — Phospholipids — Superoxide dismutase — Reperfusion — Rat

Abbreviations: SOD, superoxide dismutase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; LysoPL, lysophospholipid; TAG, triacylglycerol; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PMFS, phenylmethylsulfonyl fluoride; HPTLC, high performance thin-layer chromatography.

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Introduction

Although it is well established that cerebral ischemia due to cerebrovascular occlusion results in loss of neuronal and glial cells, the precise mechanism underlying this process remains unclear. Among the early biochemical events that have been observed following ischemia, the loss of ATP reserves, formation of free radicals and intracellular Ca^{2+} overload have been identified as critical contributors to cell damage (Bazan et al. 1992; Sims 1995; Lehotský et al. 2002). Increase in intracellular Ca^{2+} activates proteolytic and lipolytic enzymes leading to membrane protein and lipid degradation accompanied by accumulation of free fatty acids, lysophospholipids (LysoPL) and diacylglycerol (Kogure and Nakano 1992; Bonventre and Koroshetz 1993; Strosznajder et al. 1999). Membranes are also critical target sites of free radicals and lipid peroxidation is thought to play a major role in radical-mediated membrane damage during postischemic reperfusion. Depletion of ATP stores inhibits phospholipid resynthesis contributing to changes in membrane phospholipid composition (Bazan et al. 1992). These events have been proposed to affect membrane integrity and membrane-associated functions such as permeability, enzyme activities, ion transport and synaptic transmission (Parsons et al. 1997; Budd 1998; Kristián and Siesjö 1998). Changes in phospholipid composition have been documented in several studies using various experimental models of cerebral ischemia (Nakahara et al. 1991; Ishida et al. 1992; Kunievsky et al. 1992; Lukáčová et al. 1998a,b; Halašová et al. 2001). However, these studies have yielded different data on the pattern of changes and rate of lipid degradation.

The purpose of our present study was to determine phospholipid composition of rat brains subjected to 15-min ischemia or ischemia followed 2, 24 or 48 h of reperfusion. The previous study from this laboratory, using the same groups of ischemic and ischemia/reperfused animals, has shown accumulation of lipid peroxidation products, suggesting the role of an oxidative stress in brain injury. The aim of current study was, therefore, to further elucidate the relationship between free radicals and cellular injury. To assess contribution of an oxidative stress to postischemic injury we measured the activity of superoxide dismutase (SOD) – one of the major antioxidant enzymes involved in protection of nervous tissue.

Materials and Methods

Animal preparation and global ischemia

Male Wistar rats (Dobrá Voda, Slovakia) weighing 250–350 g were used in this study. Laboratory animals used in the study were maintained in accordance with institutional guidelines for animal care. Global brain ischemia was produced by four-vessel occlusion method as described (Pulsinelli et al. 1988; Murin et al. 2001). Rats were anesthetized with 2% halothane in $\text{N}_2\text{O}/\text{O}_2$ (1 : 1). During ischemia the body temperature was monitored with a rectal probe. Rats were divided into five groups: group A – control, sham operated ($n = 6$); group B – 15-min ischemia

($n = 7$); group C – 15-min ischemia and 2-h reperfusion ($n = 7$); group D – 15-min ischemia and 24-h reperfusion ($n = 9$) and group E – 15-min ischemia and 48-h reperfusion ($n = 7$).

After ischemia or required reperfusion period, the rats were sacrificed by decapitation, the heads were immediately dropped into liquid nitrogen and blood samples were taken into heparinized test tubes. The forebrain was dissected while frozen for subsequent homogenization. The tissue was homogenized for five 30-s periods at 900 rpm in 10 vol of 5 mmol/l HEPES-Tris (pH 7.4), 0.32 mol/l sucrose and 0.1 mmol/l PMFS using Potter S homogenizer (B. Braun, Melsungen, Germany).

Determination of lipids in blood plasma

Lipid concentration in blood plasma was determined enzymatically using Bio-La-Test (Lachema, Brno, Czech Republic). Plasma cholesterol was measured by enzymatic assay using Ruby Clinical Analyzer (Snijders Analysers, Tilburg, Netherlands). For analysis of phospholipids, the lipid extract of blood plasma was separated by two-dimensional high performance thin-layer chromatography (HPTLC) on silica gel 60 plates (Merck, Darmstadt, Germany). The lipidic phosphorus in phospholipid fractions was analyzed according to Bartlett et al. (1969).

Superoxide dismutase assay

SOD activity was assayed in supernatant fraction of forebrain homogenates prepared according to Dodd et al. (1981). The activity was measured using the xanthine-xanthine oxidase system for the production of superoxide and iodophenyl-nitrophenyl-phenyltetrazolium (INT) salt essentially similar to the one described by Ďuračková and Labuda (1995).

Thin-layer chromatography of brain lipids

The portion of supernatant fraction was used for preparation of lipid extract (Jungwalla and Dawson 1971). The lipids were separated by two-dimensional HPTLC. Separation of both samples and standards was performed on silica gel 60 plates (Merck, Darmstadt, Germany) according to Rouser et al. (1970). Concentrations of lipidic phosphorus in phospholipids eluted from the plates were determined as described by Bartlett et al. (1969). The phospholipid spots on silica gel plates were identified by standards (Sigma, USA) and only the spots corresponding to major phospholipids (PE, PC, PS, PI, LysoPL and SM) were used for quantification.

Statistical analysis

The results are presented as mean \pm SEM. One-way analysis of variance was first carried out to test for differences among the groups. Between groups comparisons were made using Student's *t*-test. A value of $p < 0.05$ was considered to be statistically significant.

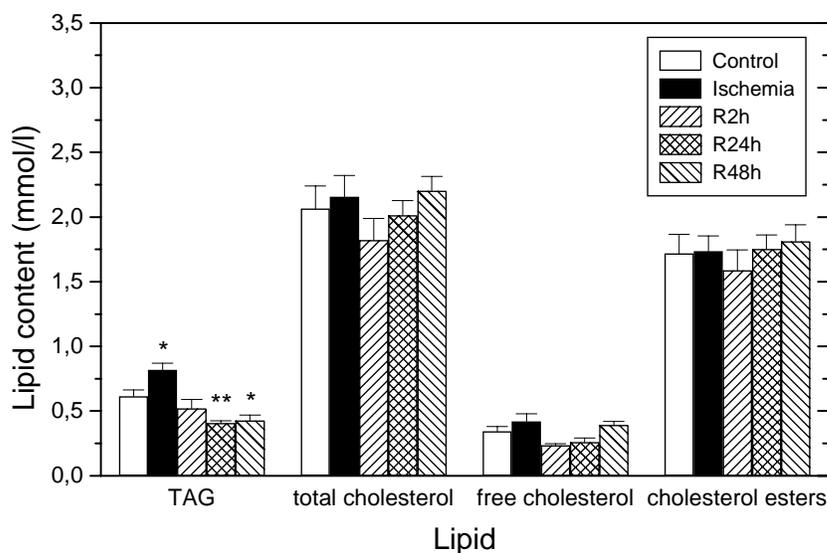


Figure 1. Effects of 15-min brain ischemia and ischemia followed by 2-, 24- and 48-h reperfusion (R2h, R24h, R48h) on the concentration of triacylglycerols (TAG) and total, free and esterified cholesterol in blood plasma. Values are given as means \pm S.E.M. of 9 animals. * $p < 0.05$, ** $p < 0.01$, significantly different as compared to control.

Results

The lipid analysis of blood plasma of rats subjected to cerebral ischemia or ischemia followed by reperfusion is shown in Fig. 1. The triacylglycerol (TAG) concentrations increased during 15-min ischemia by 33.6% of the control value, but were restored to near control value with 2-h reperfusion and after 24- and 48-h reperfusion the values were even decreased. Total and esterified cholesterol contents were unchanged during ischemia and reperfusion. Free cholesterol declined slightly during 2- and 24-h reperfusion, but was restored after 48-h reperfusion.

Fig. 2. shows the effect of ischemia and reperfusion on blood plasma phospholipids. During 15-min ischemia and 15-min ischemia followed by 2-h reperfusion the contents of individual phospholipids did not change significantly. However, prolonged reperfusion periods were associated significant decrease in phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylserine (PS) contents. After 24-h and 48-h reperfusion, the PE content, a major phospholipid component in blood plasma, decreased to $69.2 \pm 3.5\%$ and $63.3 \pm 3.6\%$, respectively. Both, PC and PS content were unchanged during 24-h reperfusion but decreased markedly after 48-h reperfusion to $48.7 \pm 4.3\%$ and $66.6 \pm 3.1\%$, respectively. Blood plasma LysoPL and sphingomyelin (SM) in ischemic and ischemia/reperfused animals did not show any significant differences.

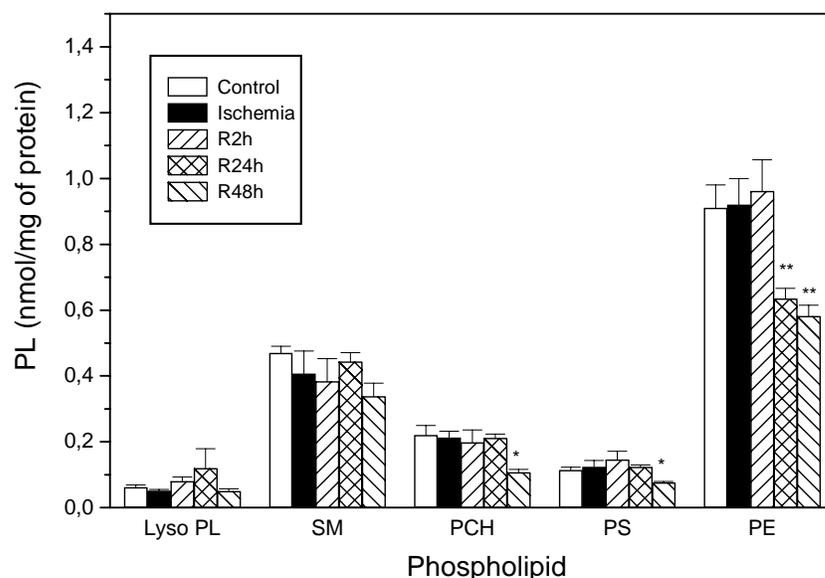


Figure 2. Effects of 15-min brain ischemia and ischemia followed by 2-, 24- and 48-h reperfusion on the concentrations of phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), sphingomyelin (SM) and lysophospholipids (LysoPL) in blood plasma. Values are given as means \pm S.E.M. of 4 animals. * $p < 0.05$, ** $p < 0.01$, significantly different as compared to control.

When forebrain phospholipids were separated by HPTLC the different profile was observed compared to that in blood plasma (Fig. 3). The major component of forebrain lipid extract is PC constituting about 49.1% of the total phospholipids. Lipid quantification has shown that PC content was markedly decreased ($60.5 \pm 5.2\%$) in ischemic rats and remained decreased during the whole reperfusion period. Forebrain contained about 35.0% of PE and only small amounts of other lipids. After 15 min of ischemia PE concentration declined by 34.1% and, similarly to PC content, was lowered during reperfusion. Phosphatidylinositol (PI) concentration was reduced by 41.1% of control value. After 2-h PI content was still significantly lower, but it rose above control value with 24–48 h of reperfusion. Ischemia induced 48.1% decrease of SM content and again, the recovery occurred only during 24–48 h of reperfusion. The PS and LysoPL concentrations were unchanged during ischemia but were 1.4- and 4.6-fold higher, respectively, after 24 h of reperfusion.

To assess the effect of ischemia and reperfusion on antioxidant defense system in brain we measured Cu/Zn-SOD activity. The activities are expressed as U/mg, where 1 U corresponds to the 50% inhibition of INT reduction by the xanthine-xanthine oxidase system. The data show that SOD activity was reduced by 20.9%

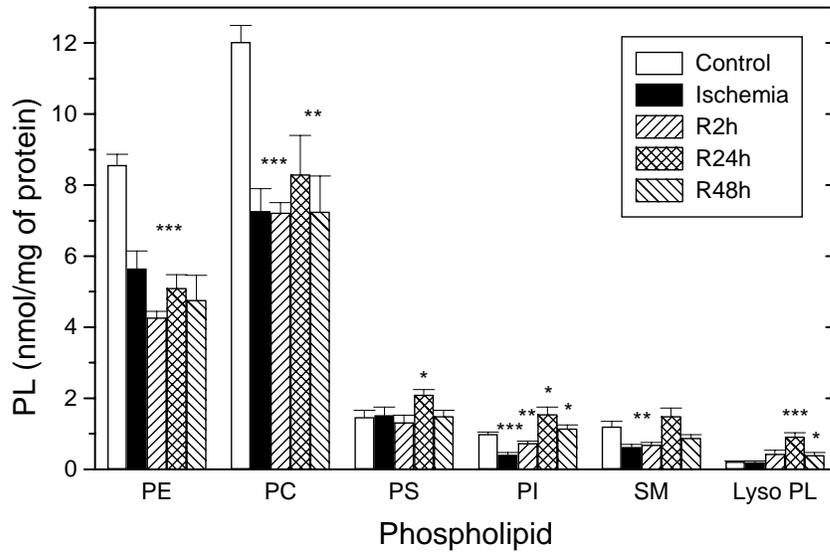


Figure 3. Phospholipid concentrations (PL) in rat brain after 15-min ischemia and ischemia followed by 2-, 24- and 48-h reperfusion. Values are given as means \pm S.E.M. of 6 animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly different as compared to control.

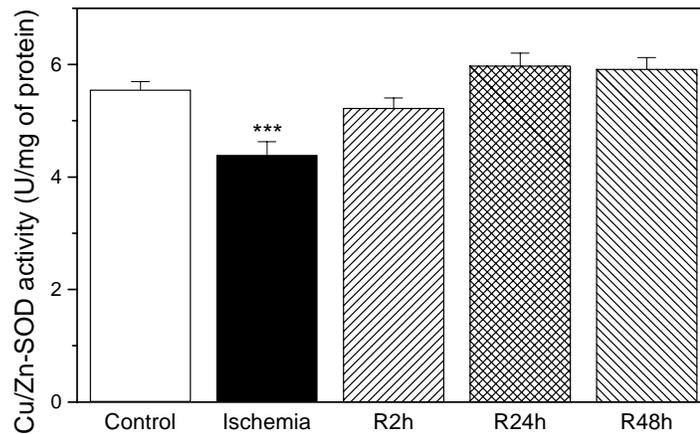


Figure 4. Cytosolic Cu/Zn-SOD activity in rat brain after 15-min ischemia and ischemia followed by 2-, 24- and 48-h reperfusion. Values are given as means \pm S.E.M. of 6 animals. *** $p < 0.001$; significantly different as compared to control.

during 15-min ischemia, however, it was restored already with short-term reperfusion period (Fig. 4).

Discussion

Present results show that short-term global brain ischemia or ischemia followed by 2, 24 or 48 h of reperfusion resulted in alterations in lipid compositions and SOD activity. We suppose that a slight increase in plasma TAG concentration might be due to a stress induced by surgical procedure and ischemia as the level recovered to control value with reperfusion. Slight decrease in plasma free cholesterol concentrations after 2 and 24 h of reperfusion can be explained by accelerated cholesterol uptake by cells, such as erythrocytes, required for regeneration of membranes damaged by ischemia and reperfusion (Solans et al. 2000).

Phospholipid analysis in rat brain after ischemia and reperfusion indicates diverse patterns of degradation of individual phospholipids. The highest degradation was observed in SM and PI fraction. The PI metabolism during brain ischemia and reperfusion has been extensively studied. In agreement with our results, the published studies have shown considerable reduction in PI content (Nakahara et al. 1991; Ishida et al. 1992; Kunievsky et al. 1992; Strosznajder et al. 1999). The ischemia-induced changes in PI metabolism are of special importance, because PI hydrolysis products inositol phosphates, DAG or arachidonic acid are recognized as second messengers playing important roles in cellular processes (Kunievsky et al. 1992; Strosznajder et al. 1999). SM metabolites – ceramide and sphingosine are also known as mediators of signal transduction (Kolesnick 2002). Hernandez et al. (2000) have shown that hypoxia and reoxygenation of cardiac myocytes activate sphingomyelinase and this activation appears to be associated with oxidative stress. Ischemia resulted also in marked decrease in PE and PC contents that continued throughout the reperfusion. Similarly, Goto et al. (1988), using four-vessel occlusion model, reported abrupt decrease in PE after 5-min ischemia, however, they did not observe any significant difference in PC level even after 60-min of ischemia. Degradation of PE has also been reported in rat brain regions after 30-min ischemia (Lukáčová et al. 1998a) and rabbit spinal cord regions after 10- or 25-min ischemic insult (Lukáčová et al. 1996, 1998b). In contrast, several investigators failed to show significant degradation of PE and other phospholipids during 3–30 min ischemic insults (Huang and Sun 1986; Nemoto and Chavko 1993). In agreement with our findings, Enseleit et al. (1994) showed marked reduction in cerebral content of SM and only small changes in PS following 15-min ischemia. However, they showed no significant changes in PE and PC concentration. The reasons for these discrepancies are not known. The variations in degradation patterns between studies may be explained by differences in animal species, experimental models or severity of ischemia. The relation between phospholipid contents in brain and blood plasma remains to be determined. Although our data suggest some similarities in changes of PE content, different pattern of changes of most phospholipids do not permit conclusions on correlation between phospholipid pools.

The decrease in PI, PE and SM contents after ischemia and reperfusion could be in part attributed to degradation of these phospholipids by activated Ca^{2+} -dependent phospholipases (Bonventre and Koroshetz 1993; Sapirstein and Bonventre 2000). Our finding that LysoPL content increases in reperfused brains supports this hypothesis. Another mechanism that may be involved in lipid degradation is free radical-mediated lipid peroxidation. Present study shows that activity of cytoplasmic Cu/Zn SOD, a scavenger of superoxide radical, significantly decreased following 15-min ischemia and recovered by reperfusion. These findings are in agreement with effects of ischemia and reperfusion on mitochondrial SOD (Sims et al. 1998). In contrast, SOD activity has been reported to remain at normal level during brain ischemia (Michowiz et al. 1990; Takagi et al. 1992). Our previously published data from the same set of experiments have shown that 15-min ischemia followed by 2, 24 or 48 h of reperfusion results in production of conjugated diene and malondialdehyde, an intermediate and end-product of lipid peroxidation (Murin et al. 2001). Present study suggests that reduced SOD activity could contribute to free radical formation and lipid peroxidation resulting in reduced phospholipid contents.

In conclusion, our study showed significant and selective reduction of brain phospholipids following ischemia and reperfusion. The present findings suggest that decreases in phospholipid concentrations may be attributable to oxidative stress and activation of phospholipases. These processes may be associated with a nonspecific increase in membrane permeability and disorders in intracellular signal transduction.

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