Ischemia/Reperfusion-induced Oxidative Stress Causes Structural Changes of Brain Membrane Proteins and Lipids

R. Murín, A. Drgová, P. Kaplán, D. Dobrota and J. Lehotský

Department of Medical Biochemistry, Jessenius Faculty of Medicine, Comenius University, Martin, Slovakia

Abstract. Oxidative stress is a recognized factor of ischemia reperfusion injury. It shares damage of lipids (LPO) and proteins (PPO), and consequently might cause changes in activity of transport systems.

Global 15 min ischemia followed by 2, 24 and 48 hour reperfusion was induced by four-vessel occlusion in Wistar rats of both sexes. Levels of TBARS and conjugated dienes as parameters of LPO were analyzed in forebrain homogenates. Concentrations of total free sulfhydryl (SH) groups and emission spectra of tryptophan were measured to quantify PPO.

Our results indicate that lipid peroxidation and protein oxidation occurs mainly during the period of reperfusion. However, significant increase in the level of conjugated dienes can be detected already after 15 min ischemia. Attack of proteins by free radicals leads to modification in structure of proteins seen as a decrease of free SH groups and tryptophan fluorescence. Ischemia/reperfusion induces formation of lipid peroxidation products as well as protein modifications.

Key words: Cerebral ischemia — Protein peroxidation — Lipid peroxidation — Rat — Fluorescence

Introduction

Ischemic brain injury is a result of a complex sequence of pathological events that evolve over time and space. Major pathogenic mechanisms of this cascade include also generation of free radicals with consequent damage of proteins, lipids and DNA.

The possible roles of free radicals in ischemic cell damage have been extensively studied since the early suggestions of Siesjo (1981). Several studies show increased formation of free radicals during ischemia induced by vessel occlusion (VO). The electron-spin resonance signal increases over 10-fold during 15 min of four-VO, measured on extracellular fluid collected in a cortical cup (Phillis and Sen 1993).

Correspondence to Prof Ján Lehotský, Ph D , Department of Medical Biochemistry, Jessenius Faculty of Medicine, Comenius University, Malá Hora 4, 036 01 Martin, Slovakia E-mail `Lehotsky@jfmed.uniba.sk`

There is a similar increase during this period in the striatum (Zini et al 1992) and in hippocampus (Piantadosi and Zhang 1996), as measured with microprobes containing a spin trap or salicylate, respectively The increase in free radicals becomes higher and unambiguous during the early reperfusion period (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)

Free radicals affect the structures of both lipids and proteins They interact strongly with unsaturated bonds in lipids, leading to a chain reaction formation of peroxides, hydroperoxides, aldehydes (Ikeda and Long 1990) and conjugated dienes (Kaplan et al 1995) Probably as a result of the formation of aldehydes and conjugated dienes, they markedly reduce membrane fluidity (Chen and Yu 1994, Kaplan et al 2000) Free radicals oxidize protein side chains, forming carbonyl groups (Freeman and Crapo 1982, Stadtman and Oliver 1991), disulfides (Freeman and Crapo 1982), and bityrosines, or leading to the loss of tryptophan (Davies et al 1987a,b) Davies et al (1987a,b) recognized oxidizing free radicals capable of provoking aggregation or fragmentation and change in electric charge Oxidized proteins show increased susceptibility to proteolytic enzymes (Davies et al 1987b) but additional alterations could be responsible both for inhibition of removal mechanisms and consequent accumulation of damaged proteins

Even quite short exposures to free radicals *in vitro* strongly inhibit many important enzymes, including plasmalemmal Na^+ pump (Elmoselhi et al 1994a,b) and both types of Ca^{2+} pumps (Racay et al 1994, 1997, 1998, Zaidi and Michaelis 1999) In case of the Na^+-K^+-ATPase, oxidation by free radicals changes enzyme activity (Matejovicova et al 1996, Kaplan et al 1997, Kurella et al 1997, Dobrota et al 1999, Lehotsky et al 1999, Rauchova et al 1999) and makes it susceptible to calpain-mediated proteolysis (Zolotarjova et al 1994)

To evaluate the role of lipid peroxidation and protein oxidation induced by transient global ischemia and reperfusion injury (IRI), the levels of conjugated dienes, thiobarbituric acid reactive substances (TBARS), free total SH-groups and fluorescence of tryptophan in forebrain homogenates were measured in the present study

Materials and Methods

Animals

Adult Wistar rats of both sexes (weighting 250–350 g) were used for study (IEP SAS Dobra Voda, Slovakia) The animals were allowed free access to food and water, and were maintained in air-conditioned room

Induction of ischemia

Forebrain ischemia was induced by the four-vessel occlusion method as described previously (Pulsinelli et al 1983) Briefly, rats were surgically prepared under halothane anesthesia by permanently occluding the vertebral arteries The carotid

arteries were exposed and loops of silastic tubing were placed loosely around them to allow rapid subsequent exposure of these vessels. The next day, the carotid arteries were located again and occluded with artery clamps. Ischemia was reversed after 15 min by removing the carotid artery clamps. 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 in ratio 1 g tissue in 10 ml homogenizing solution. Homogenate was centrifuged ($1200 \times g$ for 10 min) to remove the debris. Protein concentration was determined by the Lowry method. Control group was sham operated and homogenates were prepared as described above.

Measurements of conjugated dienes

Formation of conjugated dienes was estimated from absorbance ratio A_{233nm}/A_{215nm} of homogenate dispersed in concentration 20 μg/ml protein in solution with 10 mmol/l phosphate buffer containing 1% (w/v) Lubrol (Kaplan et al. 2000).

Fluorescence measurements of tryptophan

Both steady-state fluorescence measurements were performed in solution containing 50 μg of homogenate protein *per* ml, 10 mmol/l KCl, pH = 7.0 at 25°C in a Shimadzu RF 540 spectroflourimeter.

Fluorescence emission spectra (from 300 to 450 nm, slit width 5 nm) of tryptophan were measured with excitation at 290 nm (2 nm slit width) (Dousset et al. 1994).

Free sulfhydryl groups measurement

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

TBARS assay

Determination of TBARS formation was performed according to Das (1994). TBARS concentration was determined from the absorbance at 532 nm and expressed in nmol/g protein.

Data analysis

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

Results

Lipid peroxidation

The extent of lipid peroxidation in brain homogenate was assessed by measurement of conjugated dienes formation and changes in levels of TBARS (Tab. 1), respectively. There was a significant increase in level of conjugated dienes in groups with 15 min ischemia and 2, 24 and 48 hour reperfusion against the control, with the maximum at 24 hour reperfusion. Elevation of conjugated dienes formation during the time of 24 and 48 hours reperfusion against 15 min ischemia was also statistically significant.

Table 1. Levels of TBARS, conjugated dienes and free SH groups in rat forebrain homogenate after ischemia and reperfusion injury

	TBARS	Conjugated dienes	Free SH groups
	nmol/g	A_{233}/A_{215}	mmol/g protein
Control	378 ± 14	0 253 ± 0 003	0 34 ± 0 04
15' ischemia	409 ± 24	0 264 ± 0 003**	0 34 ± 0 03
2 h reperfusion	493 ± 45**+	0 271 ± 0 003***	0 23 ± 0 01*+
24 h reperfusion	536 ± 8***++	0 284 ± 0 002***+++	0 17 ± 0 02***+++
48 h reperfusion	550 ± 23***++	0 277 ± 0 006***++	0 20 ± 0 01**++

Values represent mean ± 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 ischemia

Ischemia in duration of 15 minutes causes no significant changes in levels of TBARS. Significant increase of TBARS was revealed in groups with 2, 24 and 48 hour reperfusion. These alterations were statistically significant compared to both the control group and the group with 15 min ischemia

Protein modification and fluorescence

Modifications in protein structure were assayed by measurement of changes in levels of sulfhydryl groups (Tab. 1) and alterations in tryptophan fluorescence (Fig 1) Ischemia in 15 min duration causes no significant changes in levels of sulfhydryl groups. But ischemia followed by 2, 24 and 48 hour reperfusion induced a significant decrease of free total sulfhydryl groups.

A significant change in fluorescence intensity of tryptophan was caused by 15 min ischemia with continuing decrease of fluorescence intensity during all the periods of the reperfusion studied.

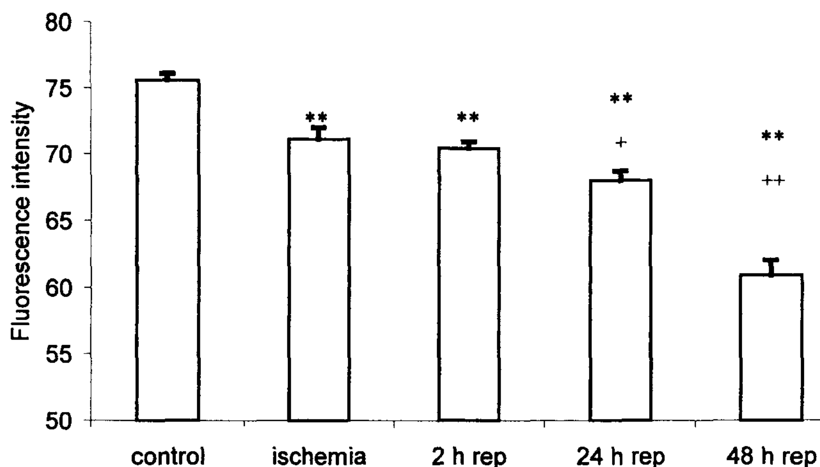

Figure 1. Effect of 15 min ischemia and various duration reperfusion on fluorescence intensity of tryptophan The results are expressed as mean ± S E M of 7 12 experiments $^{**}p < 0\ 001$, significantly different as compared to control $^{+}p < 0\ 05$, $^{++}p < 0\ 001$, significantly different as compared to ischemia

Discussion

In the present study, we investigated the effect of transient global ischemia and reperfusion on structural properties and induction of peroxidation of lipids and protein modifications in the brain.

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 presented findings demonstrate that accumulation of conjugated dienes and degradation of tryptophan can be observed as soon as after 15 min ischemia with a maximal level of conjugated dienes after reperfusion in duration of 24 hours and the whole time of reperfusion decreasing fluorescence intensity of tryptophan. After 2, 24 and 48 hours of reperfusion, when massive production of free radicals was observed in other studies (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), levels of TBARS and free SH groups were significantly changed, too.

From the experiments performed *in vitro* it is known that the effect of free radicals is rather unspecific, however, radicals strongly inhibit important enzymes regulating neuronal ion homeostasis (Matejovicova et al. 1996; Kaplan et al. 1997; Kurella et al. 1997; Dobrota et al. 1999; Lehotsky et al. 1999; Rauchova et al 1999). Oxidative alterations seen after ischemia/reperfusion could contribute to

dısturbance of neuronal ıon ıncludıng Ca^{2+} homeostasıs, whıch plays an ımportant role ın neuronal sıgnallıng (Racay and Lehotsky 1996) and consequently mıght lead to neuronal cell death (Lıpton 1999)

In conclusıon, our results suggest that ıschemıc/reperfusıon ınsult ıs assocıated wıth both lıpıd peroxıdatıon and proteın modıficatıons, whıch can be observed, ın some cases, ımmedıately after ıschemıa of 15 mın duratıon and, wıth pronounced ıntensıty, durıng tıme of reperfusıon Ischemıa/reperfusıon ınduces formatıon of lıpıd peroxıdatıon products as well as proteın modıficatıons

Acknowledgements. Presented as Abstract on the Thırd Internatıonal Symposıum on Membrane Channel, Transporters and Receptors, Smolenıce, Slovakıa, June 2000 Thıs work was partıally supported by VEGA grant No 7417/20 and Mınıstry of Educatıon grant No 123 - category C

References

Chen J J , Yu B P (1994) Alteratıons ın mıtochondrıal membrane fluıdıty by lıpıd peroxıdatıon products Free Radıcal Bıol Med **17,** 411—418

Das D K (1994) Cellular, bıochemıcal, and molecular aspects of reperfusıon ınjury Ann N Y Acad Scı **723,** 118—124

Davıes K J , Delsıgnore M E , Lın S W (1987a) Proteın damage and degradatıon by oxygen radıcals II Modıficatıon of amıno acıds J Bıol Chem **262,** 9902—9907

Davıes K J , Lın S W , Pacıficı R E (1987b) Proteın damage and degradatıon by oxygen radıcals IV Degradatıon of denatured proteın J Bıol Chem **262,** 9914—9920

Dırnagl U , Lındauer U , Them A , Schreıber S , Pfister H W , Koedel U , Reszka R Freyer D , Vıllrınger A (1995) Global cerebral ıschemıa ın the rat onlıne monıtorıng of oxygen free radıcal productıon usıng chemılumınescence ın vıvo J Cereb Blood Flow Metab **15,** 929—940

Dobrota D , Matejovıcova M , Kurella E G , Boldyrev A A (1999) Na^+/K^+-ATPase under oxıdatıve stress molecular mechanısms of ınjury Cell Mol Neurobıol **19,** 141—149

Dousset N , Ferrettı G , Taus M , Valdıguıe P , Curatola G (1994) Fluorescence analysıs of lıpoproteın peroxıdatıon Methods Enzymol **233,** 459—469

Elmoselhı A B , Butcher A , Samson S E , Grover A K (1994a) Coronary artery contractılıty, Na^+-pump and oxygen radıcals Gen Physıol Bıophys **13,** 247—256

Elmoselhı A B , Butcher A , Samson S E , Grover A K (1994b) Free radıcals uncouple the sodıum pump ın pıg coronary artery Am J Physıol **266,** C720—728

Freeman B A , Crapo J D (1982) Bıology of dısease free radıcals and tıssue ınjurv Lab Invest **47,** 412—426

Hu M L (1994) Measurement of proteın thıol groups and glutathıone ın plasma Methods Enzymol **233,** 380—385

Ikeda Y , Long D M (1990) The molecular basıs of braın ınjury and braın edema the role of oxygen free radıcals Neurosurgery **27,** 1—11

Kaplan P , Racay P , Lehotsky J , Mezesova V (1995) Change ın fluıdıty of braın endoplasmıc retıculum membranes by oxygen free radıcals a protectıve effect of sto badıne, alpha-tocopherol acetate, and butylated hydroxytoluene Neurochem Res **20,** 815—820

Kaplan P , Matejovicova M , Mezesova V (1997) Iron-induced inhibition of Na^+, K^+-ATPase and Na^+/Ca^{2+} exchanger in synaptosomes protection by the pyridoindole stobadine Neurochem Res **22,** 1523—1529

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

Kurella E , Kukley M , Tyulina O , Dobrota D , Matejovicova M , Mezesova V , Boldyrev A (1997) Kinetic parameters of Na^+,K^+-ATPase modified by free radicals in vitro and in vivo Ann N Y Acad Sci **834,** 661—665

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

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

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

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

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

Racay P , Kaplan P , Mezesova V , Lehotsky J (1997) Lipid peroxidation both inhibits Ca^{2+}-ATPase and increases Ca^{2+} permeability of endoplasmic reticulum membrane Biochem Mol Biol Int **41,** 647—655

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

Rauchova H , Drahota Z , Koudelova J (1999) The role of membrane fluidity changes and thiobarbituric acid-reactive substances production in the inhibition of cerebral cortex Na^+/K^+-ATPase activity Physiol Res (Prague) **48,** 73—78

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

Siesjo 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—51

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

Zaidi A , Michaelis M L (1999) Effects of reactive oxygen species on brain synaptic plasma membrane Ca^{2+}-ATPase Free Radical 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

Final version accepted June 20, 2001