Postischemic Reperfusion of the Spinal Cord: Prolonged Reperfusion Alleviates the Metabolic Alterations Induced by 25 min Ischemia in the Cervical and Thoracolumbal Segments

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Abstract. In recent years, increasing amount of information has indicated that in some tissues the main damage due to oxidative stress does not occur during reperfusion but during the ischemic episode of the ischemia/reperfusion event. In this respect, serious doubts were also expressed about the origin of the increased amounts of free radicals which were believed to form and reported to appear in the perfusate during the first minutes of reperfusion. Moreover, speculative explanations were only available for a second increase in lipid peroxidation which was reported to occur after postischemic reperfusions exceeding 60 min.

For this reasons, the present paper reports the results of investigation of ischemia/reperfusion injury to the cervical (CE) and thoracolumbal (ThL) segments of the spinal cord (SP) after an acute 25 min occlusion of the abdominal aorta, followed by 60–120 min reperfusion of the ischemic areas in rabbits. In CE and ThL segments of the SP, the ischemia induced: 1) a decrease in activities of superoxide dismutase (SOD), from 57.35 ± 6.36 to 45.27 ± 5.45 U.mg^{-1}.min^{-1}(S.E.M., 20.92 %), $p < 0.01$, and from 58.36 ± 5.45 to 33.00 ± 4.55 U.mg^{-1}.min^{-1}(S.E.M., 43.46 %), $p < 0.001$; 2) a significant decrease in γ-glutamyl transpeptidase (γ-GTP), from 114.66 ± 1.45 to 99.88 ± 4.4 μmol p-nitroaniline.mg^{-1}.h^{-1}(S.E.M. 12.89 %), $p < 0.05$ and from 112.24 ± 1.20 to 95.09 ± 2.40 μmol p-nitroaniline. mg^{-1}.h^{-1} (S.E.M., 16.26 %), $p < 0.05$; 3) a considerable depression in Na,K-ATPase activity, from 7.14 ± 0.58 to 5.08 ± 0.32 μmol P_i. mg^{-1}.h^{-1} (S.E.M., 28.86 %), $p < 0.01$, and from 7.23 ± 0.11 to 5.09 ± 0.31 μmol P_i. mg^{-1}.h^{-1} (S.E.M., 30.00 %), $p < 0.01$.

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The Na,K-ATPase activity became decreased by ischemia and remained depressed significantly (all $p < 0.01$) throughout the experiment. After 60 min of reperfusion, SOD activity in the CE segment and that of γ-GTP in the CE as well as ThL segments recovered, even slightly surpassing the control values, wheras SOD activity in the ThL segment became stabilized again close to its post-ischemic value. Prolonged, reperfusion for 120 min resulted in a further increase in γ-GTP activity in the CE and ThL segments (to 132.79 and 132.30 %, $p < 0.01$), and this was accompanied by a slight ($p > 0.05$) elevation in the content of conjugated dienes as well as by a new wave of depression of the SOD activity ($p < 0.05$) in both the CE and the ThL segment. From our results it could be concluded that all considerable damage to the spinal cord occurred during the ischemic period. In the period of reperfusion reparative changes started to predominate. This is in accordance with the recent discoveries indicating that, when coupled with an increase in tissue γ-GTP activity, the post-ischemic reparative changes comprise a replenishment of the cell glutathione pool. This process is accompanied with a gradual increase in H_2O_2 production that results in repeatead inhibition of the SOD activity and a tendency to conjugated dienes formation.

Introduction

There is no doubt that the degree of ischemia/reperfusion damage is always proportional to the severity and duration of the ischemic impulse (Lombardi et al. 1994, 1998). On the other hand, it has also been believed that it is the oxidative stress during reperfusion, rather than the ischemia itself, that is most damaging to the spinal cord (Braughler and Hall 1989; Kontos 1989; Butterfield et al. 1997). Hence, the question whether ischemia or the oxidative stress during the reperfusion plays the role of the killer enemy seems to be still intriguing. Nevertheless, the concept of the predominant role of free radicals that were supposed to form during the reperfusion period, could not be proven in any case. This has been recently found to concern the myocardium (Eaton et al. 1999), and it has already been directly documented in the spinal cord as well (Lukáčová et al. 1993). The main criticism against the "killer" role of a reperfusion triggered burst of free radicals is based on the finding that the tissue-accumulation of 4-hydroxy-2-nonenal (HNE), a particularly toxic product of lipid peroxidation, already culminates at the end of ischemia and that no further oxidation of lipids was found to occur during the reperfusion period (Eaton et al. 1999). HNE may react directly with amino acids in proteins, thus forming HNE-protein adducts. This introduces carbonyl groups into the proteins (Uchida and Stadtman 1992). Numerous findings indicate that HNE-protein adducts remain highly reactive, and that the late products of their interaction, which start to accumulate during reperfusion, often may be recognized erroneously as peroxidation products of free radicals created particularly in the early phases of reperfusion. For these reasons, the main interest so far has been focused on the early time intervals after the onset of reperfusion. The recent information prompted

us to review the issue of ischemia/reperfusion-induced oxidative stress in the spinal cord, with special emphasis on the events occurring during prolonged reperfusion which neurosurgeons may also be confronted with.

In the present work, ischemia/reperfusion injury to the cervical and thoracolumbal segments of the spinal cord was investigated in rabbits after an acute 25 min occlusion of the abdominal aorta, followed by 120 min reperfusion of the ischemic areas.

Materials and Methods

Treatment of animals

All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No 85–23, revised 1985, as well as with the corresponding recommendations of the respective authorities in Slovakia.

Experimental protocol

Twenty-eight male rabbits, weighing 2–2.9 kg, were anesthetized with pentobarbital (25 $mg.kg^{-1}$ i.v.). Then, a polyethylene ligature with two teflon compression knobs was placed around the abdominal aorta 1 cm below the renal arteries and it was kept loose around the closed wound. After awaking from anesthesia, the rabbits were hopping normally. Seven animals from this group served as non-ischemic controls. In 21 animals, 25 min ischemia of the spinal cord was induced by pulling the exposed ends of the ligature against the outer guidance plastic tube. This procedure made the animals fully paraplegic within 50 sec after closing the ligature on the abdominal aorta. The motor function of the forepaws and the hind legs of the experimental animals was monitored throughout the experiment. Reperfusion of the occluded area for 60 and 120 min (7 animals each) was induced by releasing the ligature. This experimental technique was essentially similar to that described by Lombardi et al. (1994), as well as in more detail in our previous paper (Lombardi et al. 1998). Tissue samples were taken quickly from the cervical (CE) and the thoracolumbal (ThL) segments of the spinal cord by a method described elsewhere (Štolc et al. 1996).

Biochemical procedures

Determination of the total (cytoplasmic & mitochondrial) superoxide dismutase (SOD) activity

Frozen samples of the spinal cord (40 mg) were homogenized in 1 ml sucrose (0.32 $mmol.l^{-1}$) at 0 ℃ and at 9500 rpm 4 times for 10 sec using an Ultra Thurrax homogenizer (Janke Kunkel, Germany). The homogenate was then sonicated twice for 20 sec, spun down at $1000 \times g$ for 10 min and, after estimation of its protein content (Lowry et al. 1953), used for the assay of SOD activity. The inhibition of superoxide-dependent cytochrome c reduction in the xanthine oxidase reaction

occurring in the presence of SOD was utilized to determine the enzyme activity (McCord and Fridovich 1969). The enzyme reaction was performed in 1.1 ml of medium (pH 7.4) containing (in mmol.l^{-1}): 20 phosphate buffer; 0.1 EDTA; 50 xanthine; 0.01 cytochrome c; and 0.05 ml cell-free tissue homogenate containing 60-80 μg protein. After 5 min preincubation at room temperature, the reaction was started by addition of xanthine oxidase (0.165 enzyme units in 15 μl). Accumulation of the reduced cytochrome c was monitored at 550 nm by means of a Hewlett-Packard diode array spectrophotometer. SOD activity was expressed as the amount of enzyme required for 50 % inhibition of cytochrome c reduction per one min.

Determination of Na,K-ATPase activity

The tissue homogenate for the determination of Na,K-ATPase activity in the spinal cord (10 % volume/weight) was prepared in a medium (pH 7.4) containing: 10 Tris-HCl; 0.5 EDTA (in mmol.l^{-1}) and 1% deoxycholate-Na salt, by using a Polytron PT-20 homogenizer (4×15 s, setting 5 at 0℃). Prior to protein and Na,K-ATPase activity determinations, the homogenate was filtered through 2 layers of gauze.

Na,K-ATPase activity was estimated by measuring the phosphate liberated from the splitting of ATP. This reaction was performed at 37℃ in 1 ml of 50 mmol.l^{-1} histidine-HCl buffer (pH 7.0), containing 2, 10 and 100 mmol.l^{-1} $MgCl_2$, KCl and NaCl respectively as well as 40–75 μg of homogenate protein. Following 10 min of preincubation, the reaction was started by adding ATP (final concentration 2 mmol.l^{-1}), and it was terminated after 10 min by adding 1 ml of ice-cold trichloroacetic acid (0.73 mmol.l^{-1}). To avoid problems that may originate from unknown and varying amounts of Na^+, K^+as well as Mg^{2+} ions brought into the reaction medium with the homogenate, the ATPase activity was estimated as the difference between the amounts of phosphate split in the absence (maximally stimulated enzyme) and presence (maximally inhibited enzyme) of 1 mmol.l^{-1} ouabain. For further details about the estimation of the Na,K-ATPase, see Ziegelhöffer et al. (1996).

Determination of γ-glutamyl transpeptidase activity

γ-Glutamyl transpeptidase (γ-GTP) activity in the homogenate was measured according to Orlowski and Meister (1970). γ-Glutamyl p-nitroanilide and methionine were used as glutamyl donor and acceptor, respectively. The rate of the reaction was measured spectrophotometrically (Specord M 40 spectral photometer, Jena Germany) at 404 nm, using the yellow color originating from the formation of p-nitroaniline. Reaction mixtures in the absence of either the substrate or the acceptor were used as reference samples.

Estimation of conjugated dienes

Frozen samples of the spinal cord were homogenized in 1 ml of ice-cold EDTA solution (15 mmol.l^{-1}) containing 4 % NaCl (solution A). Homogenization was performed similarly as described for the SOD assay. Lipids were extracted from the homogenate (0.5 ml) by a solution consisting of 0.5 ml of chloroform and 1 ml

of methanol, by vigorous mixing for 30 s. Mixing was repeated 2 times. Thereafter, 0.5 ml of solution A was added again; the whole system was mixed 2 times for 30 s and centrifuged for 10 min at 1900 × g. The procedure was essentially similar to that published by Kogure et al. (1982). Aliquots from the lower chloroform layer (0.2 ml) were taken to determine the inorganic phosphorus (Pi) according to Rouser et al. (1970). The chloroform content of the aliquot was then evaporated in a nitrogen atmosphere. Dry lipids were dissolved in an appropriate volume of cyclohexane enabling adjustment to a constant phosphorus concentration (∼ 3 ml). Absorbances at 215 nm (nonconjugated dienes) and at 233 nm (conjugated dienes) were used to calculate the increase in the content of conjugated dienes. Results were termed as the *oxidative index,* and expressed as the ratio of [Abs 233]/[Abs 215];

Protein assay

The protein content was measured according to Lowry et al. (1953).

Reagents

Chemicals used in the study were purchased from Sigma (cytochrome c, γ-glutamyl p-nitroanilide, p-nitroaliline, TRIS, ATP, xanthine oxidase), and from SERVA (methionine, chloroform, methanol, cyclohexane, bovine serum albumin, xanthine, NaCl and all other inorganic compounds).

Statistical analysis

Results were expressed as means ± S.E.M. The one-way analysis of variance (ANOVA) was first applied to test for the effect of reperfusion as a source of variance. Subsequently, the data from the experimental groups were compared with those of the sham-operated animals using the Bonferroni T test (Glantz 1981) with $p < 0.05$ considered as significant.

Results

In the present study, the content of conjugated dienes was used to indicate lipid peroxidation in tissue from CE and ThL segments of the spinal cord at the end of 25 min regional ischemia as well as after 60 and 120 min reperfusion of the occluded areas. In spite of the paraplegia developing in the animals' hind limbs, the results (Fig. 1) did not reveal any increased formation of conjugated dienes at the end of the ischemic period, or after the first 60 min of postischemic reperfusion of the occluded areas. A moderate increase in the conjugated diene content could be observed only after 120 min of reperfusion. Although the latter elevation concerned both the CE and the ThL segment of the reperfused spinal cord, it did not reach the level of statistical significance ($p > 0.05$). Ischemia induced a significant ($p < 0.001$) decrease in SOD activity in both the CE and the ThL segment of the spinal cord (Fig. 2). The decrease of SOD in the ThL segment was greater than that in the CE segment ($p < 0.01$). The more severe ischemic injury in the ThL segment persisted during the first 60 min of reperfusion. It was characterized by SOD activity 31.1 %

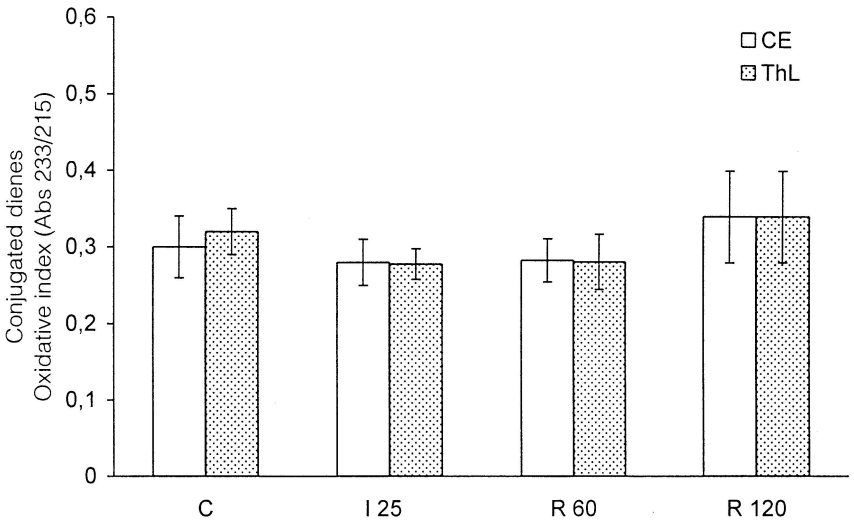

Figure 1. The ratio of conjugated dienes to non-conjugated dienes expressed as *oxidative index* (absorption at λ 233/215) in spinal cord after ischemia and reperfusion. *C* - control values at the beginning of the experiment; *I 25* – 25 min of ischemia; *R 60* – 60 min of reperfusion. Results are means ± S.E.M. expressed as the ratio of conjugated dienes to isolated double bonds (for details, see the Materials and Methods); $n = 7$. Statistical significance: $p > 0.05$.

below the control values at the beginning of the experiment ($p < 0.01$). Contrary to ThL, SOD activity in the CE segment fully recovered at the end of this period. Prolongation of reperfusion for a further 60 min reversed any tendency to recovery of SOD activities seen during the first 60 min of postischemic reperfusion. After 120 min, SOD activities decreased significantly again ($p < 0.05$) in the ThL segment. In the CE segment, SOD activity became also decreased to a level that was no more significantly different ($p > 0.05$) from any previous value measured for this segment. Hence, changes in the ThL segment proved to be more pronounced also in this case.

During ischemia, Na,K-ATPase activity became depressed ($p < 0.01$). This injury proved to be irreversible in both investigated segments since the first 60 min of reperfusion brought only a slight recovery ($p > 0.05$), while Na,K-ATPase activity (Fig. 3) completely failed to recover during the subsequent 60 min of reperfusion.

Investigation of changes in γ-GTP in the spinal cord (Fig. 4) revealed that ischemia induces a relatively moderate, but significant decrease in the enzyme's activity ($p < 0.05$). Postischemic reperfusion induced a gradual elevation in the γ-GTP activity which, after 120 min of reperfusion, reached significance at the $p < 0.01$ level vs. controls, and at the $p < 0.001$ level vs. the post-ischemic values for both the CE and the ThL segments of the SP.

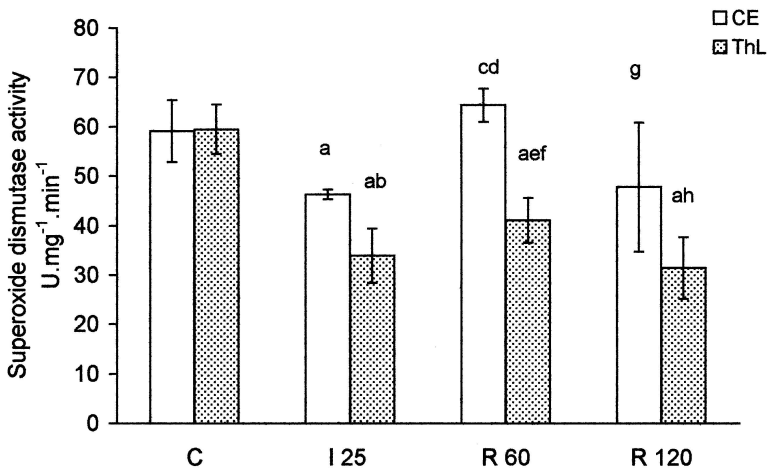

Figure 2. Superoxide dismutase activity in spinal cord after ischemia and reperfusion. *C* – control values at the beginning of the experiment; *I 25* – 25 min of ischemia; *R 60* – 60 min of reperfusion. One enzyme unit of SOD represents the amount of enzyme that is required for 50 % inhibition of cytochrome c reduction in the SOD reaction. Results are means ± S.E.M. expressed as $U.mg^{-1}.min^{-1}$; $n = 7$. For more details, see Materials and Methods. Significance: a, $p < 0.001$ vs. the corresponding controls; b, $p < 0.01$ ThL vs. CE segment at the end of ischemia; c, $p < 0.05$ vs. the value for the ThL segment at the end of ischemia; d, $p < 0.001$ vs. CE segment at the end of ischemia; e, $p < 0.01$ vs.the controls; f, $p > 0.05$ vs. ThL segment at the end of ischemia; g, $p > 0.05$ vs. CE segment in controls, *I 25* and *R 60*; h, $p > 0.05$ vs. ThL segment I 25 and R 60.

Discussion

It is well documented that ischemia causes depressions in activities of numerous tissue enzymes, including SOD and Na,K-ATPase (Röth et al. 1985; Ziegelhöffer et al. 1995; Sotníková et al. 1998). This concerns many tissues including the SP (Lombardi et al. 1998). It was shown that SOD may soon become inhibited by its own reaction product, H_2O_2 (Hodgson and Fridovich 1975). Thus, the more substrate is provided for the SOD reaction during ischemia, the earlier this inhibition may occur. An analogy drawn from cardiac tissue indicates that an enormous amount of H_2O_2 may accumulate during ischemia (Slezák et al. 1995). Another analogy drawn from the kidneys indicates that antioxidant enzymes may not be down-regulated by ischemia (Dobashi et al. 2000). In the views of the above informations it is reasonable to speculate that the significant decrease in SOD activity seen in ischemic SP prior to reperfusion may be ascribed predominantly to a feed-back inhibition of the enzyme by H_2O_2 (Hodgson and Fridovich 1975).

Many investigators have speculated that if ischemia decreases the activity of SOD the latter may become rate limiting in conversion of the superoxide anions to H_2O_2. However, thus far there is little exact evidence (based on measurements of enzyme kinetics) that SOD with ischemia-decreased activity may cope less easily

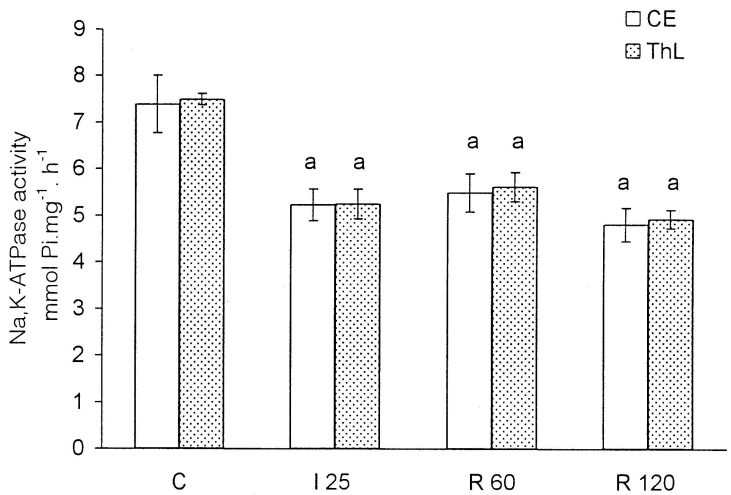

Figure 3. Na,K-ATPase activity in thr spinal cord after ischemia and reperfusion. *C* – control values at the beginning of the experiment; *I 25* – 25 min of ischemia; *R 60* – 60 min of reperfusion. Results are means ± S.E.M. expressed as μmoles of $P_i.mg^{-1}.h^{-1}$; $n = 7$. For more details, see Materials and Methods. Significance: a, $p < 0.01$ vs. the corresponding controls.

with the production of free radicals, whether it occurs during ischemia or upon the onset of reperfusion. Moreover, tissues also contain catalase. Hence, the physiological consequences of an ischemia-induced decrease in SOD activity are still far from being clear.

The dangerous consequences of a reperfusion-triggered burst of free radicals were recently seriously questioned, at least in the myocardium (Eaton et al. 1999). In this respect, we should mention that in 1989 Maršala et al. concluded that *"postischemic graded reoxygenation represents a highly neuroprotective approach in ischemia/reperfusion injury of the spinal cord"*. Hence, we are tempted to assume that, as concerns our findings, the first 60 min of reperfusion only alleviates the ischemia and/or H_2O_2-induced inhibition of SOD in SP. The repeated decrease in enzyme activity seen after 120 min of reperfusion may result from some other as yet unclarified influence, such as the accumulation of H_2O_2 induced by increased γ-GTP activity (del Bello et al. 1999), see also Fig. 4.

During ischemia, Na,K-ATPase activity was significantly inhibited (Fig. 3). This inhibition may have several reasons, with the respective mechanisms acting either individually or in cooperation: i) Na,K-ATPase may also be inhibited by calcium (Breier et al. 1998; Sulová et al. 1998) which may enter the spinal cord cells *en masse* as a consequence of ischemia-induced damage to cell membrane integrity; ii) Na,K-ATPase may also be inhibited by structural damage to the enzyme molecule itself. This is because the enzyme represents an integral part of the cell

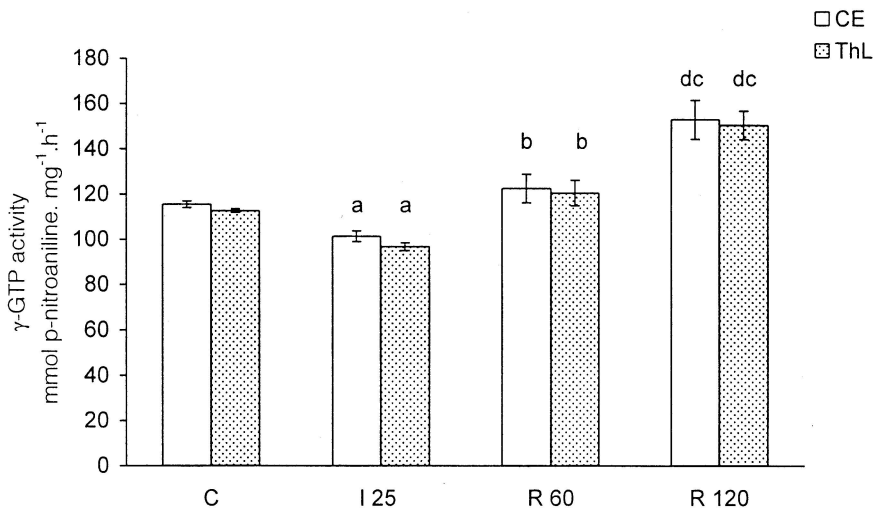

Figure 4. γ-Glutamyl transpeptidase activity in the spinal cord after ischemia and reperfusion. *C* – control values at the beginning of the experiment; *I 25* – 25 min of ischemia; *R 60* – 60 min of reperfusion. Results are means ± S.E.M., expressed as μmoles of p-nitroaniline.$mg^{-1}.h^{-1}$; $n = 7$. For more details, see Materials and Methods. Significance: a, $p < 0.05$ vs. the corresponding controls; b, $p < 0.05$ vs. the corresponding values at the end of ischemia; c, $p < 0.01$ vs. the corresponding control values; d, $p < 0.001$ vs. the corresponding values after 60 min of reperfusion.

membrane (Obšil et al. 1998) whose integrity is perturbated by ischemia (Vrbjar et al. 1994; Ziegelhöffer et al. 2000); iii) Inhibition of the Na,K-ATPase may also be caused by peroxidation of anular lipid molecules of the enzyme. This may cause a decrease in membrane fluidity, and thus in Na,K-ATPase activity (Ziegelhöffer et al. 1996, 2000); iv) Inhibition of Na,K-ATPase may also be caused by a free radical-induced attack of the essential SH-groups in the ATP binding site of the enzyme (Ziegelhöffer et al. 1983; Shattock and Haddock 1995). The resulting S-thiolated adduct may competitively inhibit the interaction of ATP in its binding site on the enzyme molecule; v) Na,K-ATPase may be inhibited by interaction of a HNE molecule with a molecule of cysteine, lysine, histidine, arginine, or proline situated somewhere whithin or close to the ATP binding site of the enzyme (Benedetti et al. 1984; Eaton et al. 1999; Ziegelhöffer et al. 2000). In our experiments, the inhibition of Na,K-ATPase in the SP was completed already during the ischemia and was neither strengthened nor alleviated by reperfusion. This indicates that any of the mentioned inhibitory mechanisms may be involved.

γ-Glutamyl transpeptidase is an ecto-enzyme that hydrolyzes the extracellular reduced glutathione (GSH) and favors the intracellular utilization of its constituent amino acids, such as cysteine to maintain normal intracellular levels of GSH and to modify the activity of GSH containing adducts (Paolicchi et al. 1997; Karp et

al. 1999). The concentration of the enzyme in tissue cell membranes may be up-regulated by nanomolar concentrations of NO (Moellering et al. 1999), as well as by oxidative stress. This points to the importance of γ-GTP in self-defence anti-oxidant mechanisms of diverse cells (Moellering et al. 1999; Chikhi et al. 1999). Nevertheless, sound evidence exists for pro-oxidative processes like superoxide and H_2O_2 production, in which γ-GTP may also be involved (Paolicchi et al. 1997; Drozdz et al. 1998). These data indicate that the γ-GTP-mediated H_2O_2 production represents a particularly important oxi-radical generating process that, via an up-regulation of the poly(ADP-ribose) polymerase concentration and activity, precedes the proteolytic cleavage of poly(ADP-ribose) induced by caspases, thus preventing even apoptosis and favoring cell proliferation (del Bello et al. 1999).

Whatever the meaning of the ischemia-induced decrese in γ-GTP activity in the SP may be, it seems more important that reperfusion did not cause any further harm to the enzyme's activity. On the contrary, the γ-GTP activity gradually increased during reperfusion. It may be speculated that the slight enhancement in the content of conjugated dienes that was noted after 120 min of reperfusion may be linked with the elevated amount of free radicals (Kogure et al. 1982) that may be generated due to the increased γ-GTP activity in this time interval. Thus, the free radicals originating from reaction mechanisms linked to γ-GTP action may be utilized in two processes that may be running parallel to one another during reperfusion: regeneration of tissues and peroxidation of lipids.

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