

The Effect of Intestinal Ischemia Duration on Changes in Plasma Antioxidant Defense Status in Rats

H. ČÍŽOVÁ, A. LOJEK, L. KUBALA, M. ČÍŽ

Institute of Biophysics, Academy of Sciences of the Czech Republic, Brno, Czech Republic

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Summary

The purpose of this study was to follow up the changes in antioxidative adaptive mechanisms induced by various periods of small intestinal ischemia in Wistar rats. The superior mesenteric artery was occluded for 15, 30, 45, 60 and 90 min. After the respective ischemic intervals, a reperfusion was set for 120 min. Samples of the serum and intestinal mucosa were taken at the end of ischemia or at the end of reperfusion. Total radical-trapping antioxidant parameter (TRAP) of the serum and the oxidative burst of neutrophils were evaluated using luminol-enhanced chemiluminescence. Individual antioxidants in the serum and the concentration of thiobarbituric acid reactive substances (TBARs) in both serum and intestinal mucosa were measured spectrophotometrically. Increased activation of circulating neutrophils was found after the reperfusion irrespective of the duration of ischemia. TRAP of the serum was increased at the end of the ischemia lasting from 30 to 90 min. This effect was further enhanced by the subsequent reperfusion period. Ascorbate and urate contributed considerably to the TRAP value especially after reperfusion following 60 and 90 min of ischemia. On the other hand, no significant changes in albumin and bilirubin serum concentrations were observed. Contrary to the mobilized antioxidative mechanisms, increased lipid peroxidation was observed in both serum and mucosa samples.

Key words

Antioxidants • Small intestine • Ischemia • Reactive oxygen species • Reperfusion

Introduction

Toxic oxygen metabolites have emerged as a major common pathway of tissue injury in a wide variety of diseases including ischemia-reperfusion (I/R) injury, because many constituents of the cell are potentially subjected to a free radical attack (Offord *et al.* 2000). Reperfusion of ischemic tissues is often associated with microvascular dysfunction. Activated endothelial cells in the microcirculation produce more reactive oxygen species (ROS) and less nitric oxide in the initial period

following reperfusion. The resulting imbalance between superoxide and nitric oxide in endothelial cells leads to the production and release of inflammatory mediators and enhances the biosynthesis of adhesion molecules that mediate leukocyte-endothelial cell adhesion (Granger 1999). Activated polymorphonuclear leukocytes (PMNL) are another important source of reactive oxygen species. PMNL are central mediators of microvascular endothelial injury in many acute pathologic processes and are a causal factor in the development of reperfusion injury (Toledo-Pereyra and Suzuki 1994). The inflammatory

mediators released as a consequence of reperfusion also appear to activate endothelial cells in remote organs that are not exposed to the initial ischemic insult. This distant response to I/R can result in leukocyte-dependent microvascular injury that is characteristic of the multiple organ dysfunction syndrome (Carden and Granger 2000).

During the evolutionary process, the organisms have developed important antioxidant defense mechanisms to withstand the constant oxidative stress. The antioxidant defense system includes small molecular antioxidants, antioxidant enzymes and metal chelating agents. Antioxidants are classified by function into four categories – preventive antioxidants, radical scavenging antioxidants, repair antioxidants, and adaptation antioxidants (Noguchi *et al.* 2000). Cellular damage occurs under the conditions where the rate of oxygen radicals is increased and/or the activity of the defense system is impaired (Nakazawa *et al.* 1996). The total antioxidant capacity of body fluids expresses a co-operative interaction between various antioxidants and is crucial for the maximum suppression of a free radical reaction in extracellular compartments (Byung 1994).

The gastrointestinal tract is one of the most sensitive tissues to ischemia and reperfusion (Mojžiš *et al.* 2001). Moreover, it is increasingly recognized as a primary effector of distant organ injury. Clinical and experimental studies suggest that oxidant species and activated neutrophils are the agents responsible for lung injury after intestinal I/R (Rossman *et al.* 1997).

Our previous studies were focused on the time course of total radical-trapping antioxidant parameter (TRAP) changes in the rat serum during different postischemic periods (Lojek *et al.* 1997, Slavíková *et al.* 1998). The whole-blood chemiluminescence (CL) was increased due to a rise in the number of polymorphonuclear cells after reperfusion in the superior mesenteric artery (SMA) occlusion/reperfusion model in rats (Hamar *et al.* 2003). Some activity of natural antioxidants was already elicited as early as at the end of the ischemic period. A further increase in TRAP was observed during the early (1-4 hours) but not during the late (1-4 days) post-ischemic period. Uric acid and ascorbic acid were found to correlate closely with TRAP, but the highest correlation was observed when yet unidentified antioxidant(s) and TRAP were compared. The purpose of this study was to obtain detailed information on the mobilization of natural antioxidant defense depending on the duration of intestinal ischemia.

Methods

Animals

Female Wistar rats (200-250 g of body weight) were maintained under 12-h light and dark cycles. Commercially available rat chow and water were provided *ad libitum*. This study was performed in accordance with the guidelines of the National Institute of Health for the care and use of laboratory animals.

Surgical procedure and sampling

The rats were anesthetized by intraperitoneal administration of ketamine/xylazine (20/2 mg per 100 g of body weight). Then the abdominal incision was performed to expose the superior mesenteric artery (SMA). Ischemia of the small intestine was set by an occlusion of the SMA. A thread was pulled around the SMA without damaging the nerves and lymphatic supply in the mesentery. The thread was then pulled through a polyethylene tube, which was immobilized in the abdominal wound following a closure of the laparotomy. The SMA remained occluded for a period specified by the animal protocol (15, 30, 45, 60 or 90 min) after which the intestine was reperfused for 0 (ischemia only) or 120 min (ischemia/reperfusion) by removing the clamp.

Blood samples obtained by a heart puncture as well as small intestinal segments from the proximal jejunum were collected at the end of the respective ischemia or ischemia/reperfusion intervals. Samples from control groups (without any surgical treatment) and sham-operated animals (midline laparotomy without occluding the SMA) were also taken at the same time intervals as for the experimental groups. Total leukocyte counts in blood samples were determined in Bürker's hemocytometer. Neutrophil counts were calculated from differential cell counts in whole-blood smears stained by the standard Pappenheim procedure.

Chemiluminescence assay of the oxidative burst of phagocytes

The oxidative burst of neutrophils was assessed by luminol-enhanced chemiluminescence using Luminometer 1251 (BioOrbit, Finland). The principle of this method was described previously (Lilius and Waris 1984). Briefly, phagocyte-derived oxidizing species interacted with luminol and the resulting light emission was measured at a wavelength of 425 nm. The luminometer was set to measure 25 samples for a period of 65 min. The temperature was maintained at 37 °C. The

samples contained 2.5 µl of whole blood, 50 µl of 10mM luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione; Sigma, USA) in borate buffer and 50 µl of opsonized zymosan (2.5mg/ml; Sigma, USA). The total volume of 600 µl was attained by adding 500 µl of Hank's balanced salt solution (HBSS, pH=7.4). The CL values were expressed as integrals of the obtained kinetic curves corresponding to the total amounts of light produced during the time of the measurements. Spontaneous chemiluminescence without any activator was also measured in all samples. Solutions used were tested in order to eliminate any phagocyte activating or inhibiting effects.

Chemiluminescence TRAP analysis

The principle of this method was described previously (Uotila *et al.* 1994). The CL signal is driven by the production of luminol-derived radicals generated from a thermal decomposition of 2,2-azo-bis-2-amidinopropane hydrochloride (ABAP) purchased from Polyscience, USA. The reaction mixture contained 475 µl of PBS (100mM, pH = 7.4), 50 µl of 10 mM luminol in 100 mM borate buffer (pH = 7.4) and 20 µl of a serum sample. The cuvettes were incubated at 37 °C in a temperature-controlled carousel of the luminometer for 10 min. To start peroxy radical generation, 50 µl of 400 mM ABAP were added. The TRAP value was determined from the period of time, during which the serum samples quenched the CL signal due to the present antioxidants. A water-soluble analogue of tocopherol, Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; Aldrich, USA) was used as a reference inhibitor in a concentration of 8 nM.

Individual antioxidant measurement

The concentrations of urate, albumin and bilirubin were determined spectrophotometrically by using commercial Bio-LA-TEST kits (Lachema, Czech Republic). Serum samples (100 µl) for ascorbic acid determination were stabilized with ice-cold 10 % trichloroacetic acid (400 µl) and centrifuged (20000 xg, 5 min, room temperature). The supernatant was used for the spectrophotometric detection based on the reduction of ferric chloride to ferrous ion by ascorbic acid and a further reaction with 2,4,6-tripiridil-S-triazina (TPTZ; Sigma, USA) developing a violet chromophore. The reaction mixture consisted of 125 µl of 0.048 M TPTZ dissolved in 0.1 N HCl and 100 µl of 0.02 M FeCl₃ in 0.2 N HCl, the final volume was adjusted to 25 ml with

acetate buffer (12.6 ml of 5 M acetic acid, 100 ml of 5M sodium acetate, 29.2 ml of methanol). Then 200 µl of the reaction mixture was mixed with 80 µl of a serum sample or a standard (ascorbic acid 8 µg/ml in TCA). The absorbance at 595 nm was measured after 5 min of incubation at 20 °C.

Lipid peroxidation

The concentration of thiobarbituric acid reactive substances (TBARS) was used as the index of lipid peroxidation as described previously (Slavíková *et al.* 1998). Briefly, 1 or 2 ml of the reaction mixture consisting of 0.6 % thiobarbituric acid (Sigma, USA) and 1 % phosphoric acid (Lachema, Czech Republic) at a ratio of 1:3 (v/v) were added to the serum (0.1 ml) or tissue homogenates (0.05 g of tissue in 450 ml of PBS), respectively. The samples were incubated in a water bath (100 °C) for 45 min. After being cooled to laboratory temperature, 1 ml of *n*-butanol was added and the mixture was shaken vigorously. The samples were centrifuged (5 min, 2000 xg) and the absorbance of the upper layer was measured at 532 nm. 1,1,3,3-tetraethoxypropane (Sigma, USA) at a final concentration of 0.1 µM was used as a standard. Lipid peroxidation was expressed in nmol of TBARS per 10 mg of the tissue or 1 ml of the serum.

Statistical Evaluation

All data are expressed as mean ± S.E.M., n = 8. The data were analyzed by one-way analysis of variance (ANOVA) followed by Student's *t*-test with a level of significance of p<0.05. Pearson correlation coefficient (*r*) was used to evaluate correlations among TRAP and individual small molecular antioxidants and linear regressions were calculated.

Results

Total leukocyte counts increased with a prolonged duration of ischemia followed by 2 h of reperfusion (Fig. 1a). The increase in leukocyte count was caused mainly by a significant mobilization of circulating neutrophils after ischemia/reperfusion (Fig. 1b). There was a significant increase in the total chemiluminescence response of whole blood phagocytes activated by opsonised zymosan at all intervals of ischemia followed by reperfusion (Fig. 2a). The CL response corrected for 10³ neutrophils did not change significantly after different periods of ischemia alone. On

the other hand, when the ischemia was followed by 2 h of reperfusion, CL response corrected for 10^3 neutrophils decreased significantly from 30 min of ischemia (Fig. 2b). The spontaneous CL response did not change significantly (data not shown).

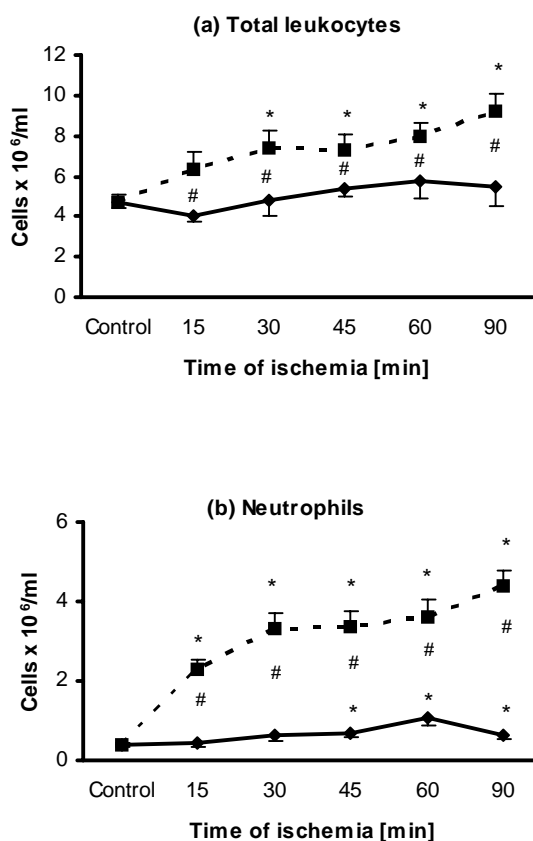


Fig. 1. Changes in total leukocyte (a) and neutrophil (b) numbers in rats with intestinal ischemia (15-90 min) and reperfusion (120 min). Dashed lines represent values of the groups with ischemia followed by reperfusion, solid lines represent groups with respective ischemia only. * - significant ($p \leq 0.05$) changes in a given parameter compared to the pre-operative value. # - significant ($p \leq 0.05$) differences between the corresponding time-points of the ischemic and ischemia/reperfusion groups.

The total peroxyl radical trapping antioxidative parameter value of serum samples was significantly increased at the end of ischemia intervals from 30 min to 90 min when compared to pre-operative levels. The effect of ischemia was further enhanced by a subsequent reperfusion period after the last two intervals (60 and 90 min) of ischemia (Table 1).

Serum concentrations of ascorbate increased significantly after ischemia at all sampling points and this increase was further enhanced by subsequent 2 h of reperfusion (Fig. 3a). Serum concentrations of uric acid were significantly increased during the whole ischemia

period. After ischemia followed by reperfusion a significant elevation was found only at 45 and 90 min of ischemia/reperfusion (Fig. 4a).

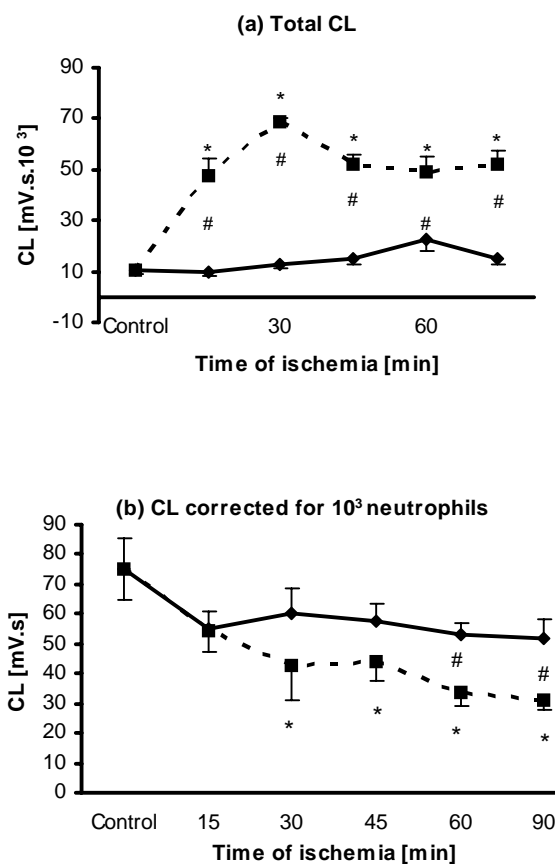


Fig. 2. Changes in total CL (a) and CL corrected for 10^3 PMNs (b) activated by opsonized zymosan in the whole blood in rats with intestinal ischemia (15-90 min) and reperfusion (120 min). For symbols and explanations see text to Fig. 1.

Table 1. Total radical-trapping antioxidant parameter (TRAP) of the serum in rats with intestinal ischemia (15-90 min) and reperfusion (120 min).

	TRAP ($\mu\text{mol.l}^{-1}$)	
	I	I/R
Control	519.4±25.4	
15 min	589.0±28.2	636.4±31.0*
30 min	720.5±55.1*	631.1±37.2*
45 min	707.6±27.9*	599.3±30.7
60 min	784.0±60.3*#	1195±172*
90 min	791.4±62.0*#	2213±155*

Data are expressed as the mean \pm S.E.M., $n = 8$. Asterisks show statistically significant changes ($p \leq 0.05$) in a given parameter compared to the pre-operative control value. Double crosses show significant differences ($p \leq 0.05$) between the corresponding time-points of the ischemic and ischemia/reperfusion groups.

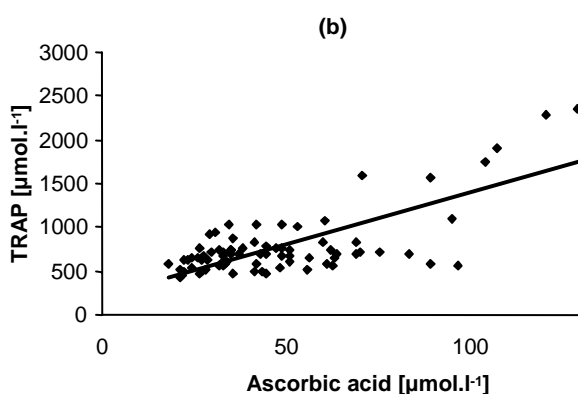
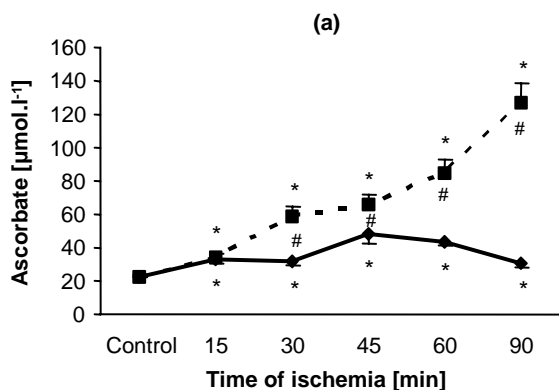


Fig. 3. Changes in the serum concentration of ascorbic acid (a) and a correlation between ascorbic acid serum concentration and TRAP (b) in rats with intestinal ischemia (15-90 min) and reperfusion (120 min). For symbols and explanations see text to Fig. 1.

Ascorbic acid and uric acid contributed considerably to the TRAP value especially after the reperfusion following 60 and 90 min of ischemia. When TRAP values were correlated against the serum concentrations of these two antioxidants, the correlation coefficients for ascorbic acid and uric acid were $r = 0.75$ (Fig. 3b) and $r = 0.88$ (Fig. 4b), respectively.

Serum concentrations of albumin and bilirubin did not change significantly (data not shown). Contrary to the mobilized antioxidative mechanisms, increased lipid peroxidation in both mucosa and serum samples was observed (Table 2).

There was also a non-significant increase in phagocyte mobilization in the sham-operated group. No time-dependent changes were found in individual antioxidant concentrations (ascorbate and urate) and in the TRAP value. No changes in lipid peroxidation were observed either in mucosal or serum samples (data not shown) in the sham-operated group.

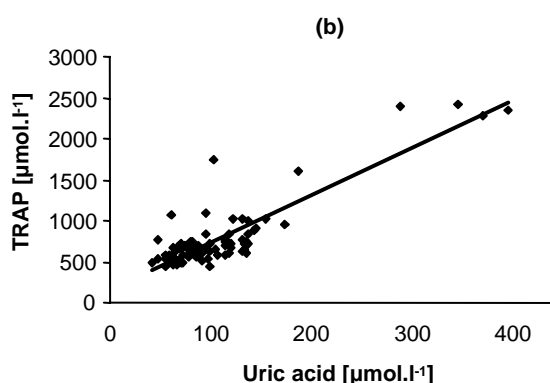
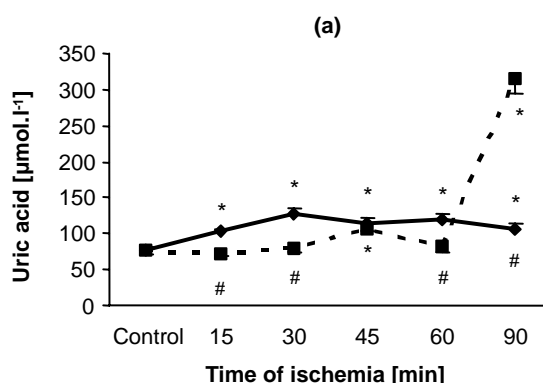


Fig. 4. Changes in the serum concentration of uric acid (a) and a correlation between uric acid serum concentration and TRAP (b) in rats with intestinal ischemia (15-90 min) and reperfusion (120 min). For symbols and explanations see text to Fig. 1.

Discussion

Intestinal ischemia-reperfusion injury is still highly investigated for its serious clinical relevance especially in intensive care patients (Schwarz *et al.* 1999). It can be shown that the morphogenesis of ischemic damage to the intestinal mucosa in man does not differ from that in the rat despite the differences in the time course of the ischemic damage (Wagner and Gabbert 1983). In our work, extracellular antioxidant status was studied as a function of ischemia duration to evaluate the hypothesis that the severity of ischemia plays a determining role in the extent of oxidative damage in rats.

The amount of reactive oxygen species produced is under the control exerted by antioxidant defense mechanisms. Complete elimination of ROS is not possible considering their physiological role as a signal, messenger and trigger molecules (Nordberg and Arnér 2001). It has been reported that mild oxidative stress

upregulates the antioxidative status of tissues. For example, Porreca *et al.* (1994) found that the longer the duration (10-120 min) of myocardial ischemia, the greater the imbalance between the myocardial antioxidant system and free radical aggression. Longer intervals of intestinal ischemia induce tissue damage. Lehmann *et al.* (1995) observed an increase in malondialdehyde as a marker of lipid peroxidation after 60 min reperfusion in rats with the superior mesenteric artery occluded for one hour. To establish the antioxidant status of rat intestinal tissues after ischemia-reperfusion the antioxidant enzymes were studied by Kaçmaz *et al.* (1999). Superoxide dismutase and glutathione peroxidase activities were reported to decrease, but the catalase

activity and TBARS level increased. Thus the enzymatic antioxidant defense system was significantly changed after ischemia-reperfusion and intestinal tissue was exposed to increased oxidative stress, the results of which were the peroxidation in some cellular structures and increased concentrations of oxidative products. Osborne *et al.* (1994) determined intracellular antioxidant enzymes and they concluded that the development of oxidant tolerance in the small intestinal mucosa does not involve the active participation of the epithelial lining being located in the lamina propria. On the other hand, Grisham *et al.* (1990) showed a low enzymatic antioxidant activity in human colonic mucosa being localized within the epithelium but not in the mucosal lamina propria.

Table 2. TBARS as a measure of lipid peroxidation in the intestinal mucosa and serum samples of rats with intestinal ischemia (15-90 min) and reperfusion (120 min).

	TBARS in serum (nmol/ml)		TBARS in intestinal mucosa (nmol/10 g)	
	I	I/R	I	I/R
<i>Control</i>	1.56±0.21	2.09±0.23		
<i>15 min</i>	4.81±0.45* [#]	1.00±0.49	3.66±0.66* [#]	2.12±0.10
<i>30 min</i>	3.78±0.36*	3.59±0.45*	3.10±0.46*	5.26±1.11*
<i>45 min</i>	2.75±0.99	5.84±1.35*	3.87±0.53*	3.28±0.47*
<i>60 min</i>	2.39±0.74	4.82±1.64*	3.45±0.66*	2.79±0.27
<i>90 min</i>	2.19±1.37 [#]	4.79±0.87*	4.89±1.17* [#]	2.32±0.41

Data are expressed as the mean ± S.E.M., n = 8. Asterisks show statistically significant changes ($p \leq 0.05$) in a given parameter compared to the pre-operative control value. Double crosses show significant differences ($p \leq 0.05$) between the corresponding time-points of the ischemic and ischemia/reperfusion groups.

It was observed in our experimental model that the total antioxidative capacity of serum from animals with ischemic/reperfused small intestine was increased at the end of ischemia intervals from 30 to 90 min when compared to pre-operative levels. The effect of ischemia was further enhanced by a subsequent reperfusion period after the last two intervals of ischemia. Due to the short induction phase, it can be assumed that this increased antioxidant capacity of serum is independent of protein synthesis and is not associated with changes in the activity of endogenous antioxidant enzymes, which are mostly linked to the intracellular milieu. Thus, already synthesized and/or dietary chain-breaking antioxidants released into the serum from their tissue deposits should play a major role in inducing systemic tolerance to oxidative stress in circulation.

As TRAP measures the total peroxy radical trapping capacity, practically all chain-breaking

antioxidants and their interactions in a fluid sample are included, while the measurement excludes other kinds of antioxidants, e.g. those acting to prevent the initiation of the peroxidation process by sequestering a transition metal catalyst. Originally, plasma TRAP was reported to be composed of uric acid, ascorbic acid, vitamin E and protein sulfhydryl groups, but there may still exist a substantial unidentified antioxidant component comprising 25-35 % of total TRAP (Uotila *et al.* 1994). In our experiments, the increase in total antioxidant capacity was accompanied with an increase in urate and ascorbate serum concentrations. On the other hand, no changes were observed in the serum concentrations of albumin and bilirubin.

One of the most likely candidates contributing to the increased antioxidant status of the serum is urate produced in purine catabolism by xanthine oxidoreductase. This enzyme is converted during

ischemia from its dehydrogenase to its oxidase form and after reoxygenation, it employs molecular oxygen as an electron acceptor playing a dual role in the redox status in ischemic/reperfused tissues (Kooij 1994). The intestinal tissue is reported to exert a very high activity of xanthine oxidoreductase (Schiller *et al.* 1993). Moreover, it was observed that rats subjected to intestinal I/R had an increased plasma xanthine oxidase activity (Terada *et al.* 1992). From the other constituents of TRAP, ascorbic acid contributes to the total antioxidant capacity to a minor extent due to its very low concentration in the serum compared with urate as was reported for man (Uotila *et al.* 1994). However, one must take into consideration that rats are able to synthesize ascorbic acid. Thus, we cannot exclude a role of ascorbic acid in the increased antioxidant status of serum in the post-ischemic period. During its antioxidant action, ascorbic acid undergoes a two-electron oxidation to dehydroascorbic acid, which is relatively unstable and can be reduced back to ascorbic acid in erythrocytes and other blood cells (Stocker and Frei 1991). In addition to scavenging oxygen radicals directly, uric acid could stabilize ascorbic acid in serum (Sevanian *et al.* 1985). The concentration of ascorbate is likely to be a result of both its synthesis *de novo* in rats (there is a marked increase in its concentration only 2 h after reperfusion), its recycling in blood cells and its stabilization in serum with the help of urate.

Albumin, the most prominent of plasma proteins, is provided with 17 disulfide bridges. The single remaining cysteine residue is thought to contribute to the plasma sulfhydryls that react with peroxy radicals (Wayner *et al.* 1985). Lindeman *et al.* (1989) have shown that bilirubin could contribute to the TRAP only to a minor extent. It was suggested in our previous study that other antioxidants are included in the phenomenon observed in our experiments (Lojek *et al.* 1997). Compounds like β -carotene and flavonoids could also contribute to the total antioxidative activity of serum samples. However, Lindeman *et al.* (1989) found the plasma concentration of β -carotene too low to make a significant contribution to TRAP. Neither can flavonoids

be responsible for a large proportion of the activity of unidentified antioxidants. It can be concluded that plasma contains one or more important antioxidant(s) that remain to be identified (Aejmelaeus *et al.* 1996).

Despite the mobilized antioxidative mechanisms, there was a significant increase in the total chemiluminescence response of whole blood phagocytes activated by opsonized zymosan and an increased lipid peroxidation in both the mucosa and serum samples was also observed. Since the CL response corrected for 10^3 neutrophils did not change significantly after different periods of ischemia alone, and it even decreased significantly when the ischemia was followed by 2 h of reperfusion, neutrophil mobilization rather than their activation was responsible for the increased reactive oxygen species production. It seems that in the case of reperfusion, the produced ROS are eliminated to a small extent due to the increased TRAP. On the other hand, the increase in the TRAP of serum after ischemia/reperfusion was not sufficient to prevent lipid peroxidation, probably due to the dual role of two major constituents of TRAP in the redox status (Benzie and Strain 1996).

It can be concluded that the ischemia of rat small intestine enhanced the activity of natural antioxidant mechanisms of the serum depending on the duration of ischemia and on the presence or absence of subsequent reperfusion. The increase in the total antioxidant capacity of the serum was not sufficient to prevent lipoperoxidation either in serum or the intestinal tissue. Further studies are needed to clarify the contribution of individual antioxidants to the changes in the antioxidant status of serum and to compare the activity of extracellular and intracellular antioxidants to evaluate the complex antioxidant status of the body.

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Reprint requests

Milan Číž, Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, 612 65 Brno, Czech Republic, fax: +420 541 211 293, e-mail: milanciz@ibp.cz