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Time Course of Leukocyte Response and Free Radical Release in an Early Reperfusion Injury of the Superior Mesenteric Artery

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Summary

The sequence of changes in circulating immune cells and in free radical production was studied during the small intestine reperfusion. Rat small intestine ischemia/reperfusion was induced by a 45 min superior mesenteric artery occlusion followed by a 4-hour reperfusion. Samples of peripheral blood were collected every 20 min during reperfusion. While the number of polymorphonuclear leukocytes increased significantly both in the sham-operated controls and the experimental group (about 400 % at the end of reperfusion), a decrease in lymphocyte counts to 60 % was observed in the experimental group only. Although there were no changes in the counts of total T lymphocytes, a significant reduction in B cell counts was observed. Flow-cytometrical measurements showed no changes in the Tc subpopulation, while the Th subpopulation increased in the experimental group only. Free radical generation in blood (luminometric measurements) increased gradually and reached an eight-fold level by the end of reperfusion in both groups. Thus, it has been shown that the increase in free radical production is mainly due to the increased number of polymorphonuclear leukocytes mobilized already at the initial stages of reperfusion. The reduction in B lymphocyte population is probably due to homing mechanisms.

Key words

Rat • Polymorphonuclear leukocytes • Lymphocytes • Chemiluminescence

Introduction

Severe intestinal hypoxia or ischemia followed by reperfusion causes a destruction of the gut mucosa (Chiu *et al*. 1970, Haglind *et al*. 1980, Illyés and Hamar 1992). Reperfusion of the gut results in a release of reactive oxygen species (ROS) which, among other factors, are responsible for the mucosal damage. The damaged vascular surface is a source for ROS production (Granger *et al*. 1981). Moreover, inflammation (Otamiri 1989) and tissue regeneration (Illyés and Hamar 1992) processes following reperfusion attract immune cells, especially polymorphonuclear leukocytes (PMNL), which also contribute to ROS production (Granger 1988, Schoenberg *et al*. 1984). ROS, in turn, recruit further PMNL which induce additional tissue damage when activated (Schoenberg *et al*. 1984, Grisham *et al*. 1986, Otamiri 1989). The sequence and extent of these

processes at an early period of reperfusion play a significant role in determining organ and vascular functions following reperfusion. The sequence of events in immune cell activation and ROS release with respect to PMNL function and mucosal damage has not yet been established.

Several methods have been developed to assess changes in PMNL activation. One of them involves chemiluminescence (CL) measurements of the granulocyte-derived ROS during the oxidative burst of cells (Allen *et al*. 1972, Lilius and Marnila 1992). Číž and Lojek (1993) and Lojek *et al*. (2002) have modified this assay to quantify the total CL activity of the whole blood under different experimental conditions.

In earlier studies, we observed some characteristic changes in the profile of the circulating leukocytes during intestinal ischemia/reperfusion. These studies showed little changes in total leukocyte counts accompanied by a decrease in lymphocyte and an increase in granulocyte counts (Hamar *et al*. 1994, Lojek *et al*. 1997b).

The present study was aimed at analyzing in detail the reactions of the immune cells and the phagocyte-derived generation of ROS in intestinal ischemia/reperfusion experiments during early stages of reperfusion. Changes in cell counts (leukocytes, lymphocytes and PMNL) and the CL of the whole blood were measured every 20 min for 4 hours after the start of reperfusion. To evaluate the changes in lymphocyte subpopulations, immunophenotyping of the lymphocytes was also carried out.

Methods

Animals, anesthesia and surgical procedures

Male Wistar rats (3 months old, 250-300 g body weight) were kept under controlled light and temperature conditions. They were allowed free access to water and food before the experiments. The rats were subjected to gut ischemia and reperfusion as described below. These animal studies conformed with the Hungarian Law of Animal Protection.

Animals were anesthetized by an intramuscular administration of ketamine and xylazine (20 and 2 mg per 100 g of body weight, respectively). Maintenance doses of anesthetics were given every hour. The surgical procedures were carried out under sterile conditions.

The left external jugular vein was cannulated for the injection of solutes. Another cannula was introduced into the right carotid artery for blood collection. Following midline laparotomy, the small intestine was exteriorized and a thread was pulled around the superior mesenteric artery (SMA) without damaging the nerves and lymphatic supply in the mesentery. The thread was pulled through a polyethylene tube, which was immobilized in the abdominal wound following suture of the laparotomy incision. After 45 min of ischemia the superior mesenteric artery was reperfused. The rats were kept under anesthesia, their body temperature was maintained by a heating pad, and they were allowed to breathe spontaneously.

Chemiluminescence assay

Luminol-enhanced chemiluminescence of the whole-blood cells was measured with a 1251 type luminometer (BioOrbit, Finland). The details of the procedure have been published elsewhere (Lojek *et al.* 1997a). Each sample luminescence was measured 20 times at intervals of 201 seconds. The temperature was kept at 37 °C. The activated samples contained 5.0 µl blood, 50 µl 10^{-2} M luminol (Sigma) in a borate buffer, 100 µg zymosan particles (Sigma) opsonized with rat serum, and Hanks balanced salt solution to reach a total volume of 500 µl. The buffers and Hanks solution were verified not to modify either phagocytosis or chemiluminescence. All assays were run in triplicate. Spontaneous CL (without zymosan) was also measured. CL values were expressed as an integral of the obtained kinetic curves corresponding to the total amount of light produced during the measurements.

Experimental protocol

The animals were randomly allocated into two groups (n=10): sham-operated and experimental. The sham-operated animals underwent laparotomy and the preparation of the superior mesenteric artery only, without occlusion. Following surgery, 1500 U/kg of heparin were administered. Control blood samples were withdrawn prior to the occlusion of the superior mesenteric artery. Blood samples from experimental and sham-operated controls were withdrawn at the end of the occlusion and 45 min after the operation, respectively. Later, samples were collected every 20 min after the onset of reperfusion, which lasted for 4 h, or at the respective time intervals in the sham-operated group. Arterial blood samples were collected into a glass capillary (250 µl each time) for blood gas analysis (Compact 1 Blood Gas Analyzer, AVL, Austria), hematocrit (Htc), cell counts, and chemiluminescence determinations. Blood gases and Htc were determined

only every 60 min. The total number of leukocytes was measured in a Bürker's chamber, and the qualitative composition of lymphocytes and PMNL was evaluated in blood smears. Because of the significant hemo-concentration during reperfusion (Lojek *et al.* 1997b), a Ringer-lactate solution was infused: 5 % of body weight during the first, and 2.5 % during the second and third hours of reperfusion, respectively. Sham-operated animals were administered the same volume of fluid. The fluid was manually administered with a sterile syringe: 1 ml of Ringer-lactate solution was given to a 300 g rat every fourth minute during the first hour and 0.5 ml during the second and third hours, respectively.

Lymphocyte immunophenotyping

In a separate series of studies, arterial blood (1.5 ml) was collected from 10 sham-operated and 10 experimental rats in heparinized tubes before the SMA occlusion, at the end of 1 and 4 h of reperfusion. The samples were analyzed within one hour. Anti-rat antibodies and reagents for the flow cytometric analysis were purchased from Serotec. T cells were identified with an anti-CD2 antibody; B cells were characterized with an anti-CD19 antibody. Phycoerythrin-labeled anti-CD4 and fluorescein isothiocyanate-labeled anti-CD8 were used for double labeling of lymphocytes to quantify Th/i and Tc/s subpopulations, respectively. The phenotyping of the immune cells was carried out in a Coulter Epics Elite flow cytometer (wave length: 488 nm). The Elite 4.01 software was used for the analysis.

Statistical analysis

Data are expressed as the mean ± S.E.M. All changes are presented as % of the preocclusion control values. Data analyses were carried out using the Statgraphics program package. Either one-way or multiple analyses of variance (ANOVA), with Bonferoni *post hoc* test were used. $P<0.05$ values were considered as significant.

Results

Blood gas, pH and hematocrit values did not change in the experimental group. In the sham-operated group, blood gases and pH were also within the physiological range, but hematocrit decreased from 44 % to 37 % as a result of the infusions. Total leukocyte counts were 8.15±0.12 x 10^3/μl (71.5 % lymphocytes, 28.5 % PMNL) at the beginning of the studies.

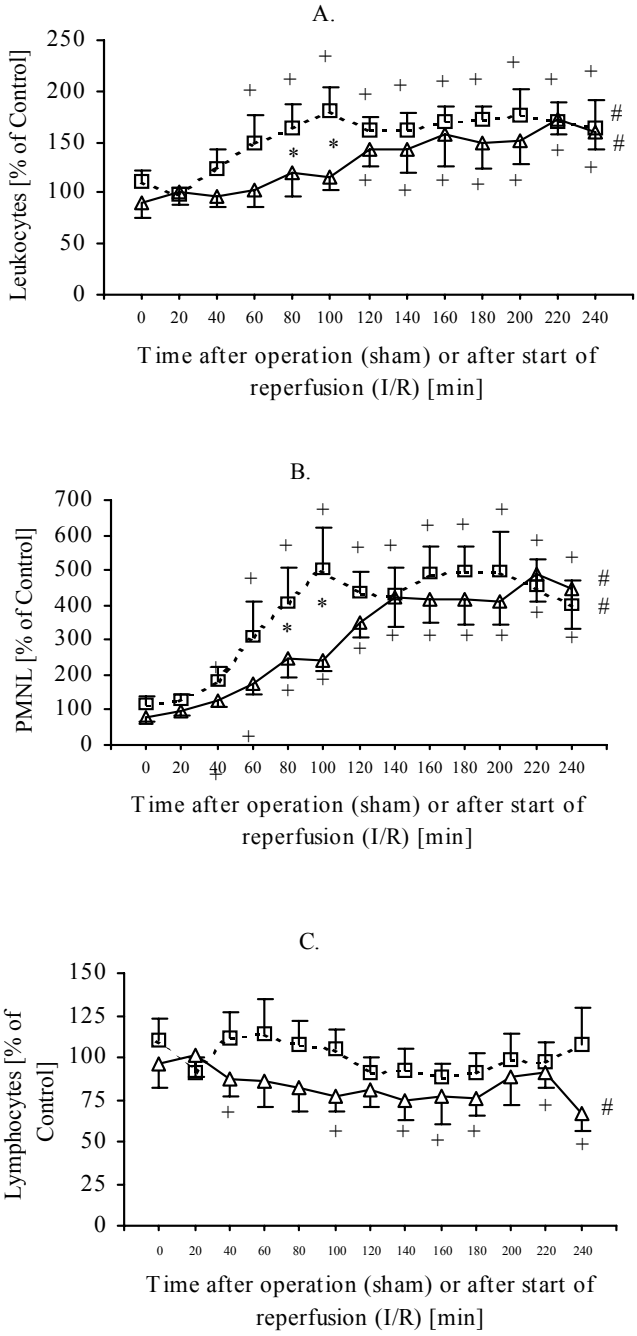

Fig. 1. *Changes in leukocyte (A), PMNL (B), and lymphocyte (C) counts during a 4-hour reperfusion of rat small intestine. Solid line: experimental group, broken line: sham-operated group. Data represent relative values compared to postoperative control (100 %) levels. Symbol + indicates significant differences compared to the preischemic value in the experimental group, and compared to the postoperative values in the sham-operated group; symbol * indicates significant differences between given time points of the two groups (sham vs. experimental); symbol # indicates a significant change in the given parameter (ANOVA).*

The number of leukocytes significantly increased both in the sham-operated controls and the experimental group. However, the increase of leukocytes started one hour earlier in the sham-operated group. At the 100th min of reperfusion this increase rose by 76±26 and 16±24 % in the sham-operated and experimental groups, respectively (Fig. 1A). Compared to the controls, PMNL counts increased both in the sham-operated and experimental groups starting from the 40th min of reperfusion. The increase amounted to 500±107 % in the sham-operated and 235±22 % in the experimental groups after 100 min of reperfusion (Fig. 1B). Lymphocyte counts significantly decreased during reperfusion in the experimental group, whereas no significant changes were observed in the sham-operated group (Fig. 1C).

The spontaneous chemiluminescence did not differ from the background value either in the sham or the experimental groups at any time. CL was within the range of 1000-1500 mV•s. Stimulation with zymosan increased the total CL up to 13971±1155 and 11148±680 mV•s in the sham-operated and experimental groups, respectively. CL stimulated by zymosan increased significantly in both groups after 60 min of reperfusion. CL was significantly higher in the sham group between the 100th and 140th min of reperfusion (Fig. 2). The increase in stimulated CL corresponded to the increase in the PMNs. CL at each individual measuring point was also corrected for 10^3 PMNs. There was no significant change in the corrected signal as a function of time in any of the two groups (data not shown).

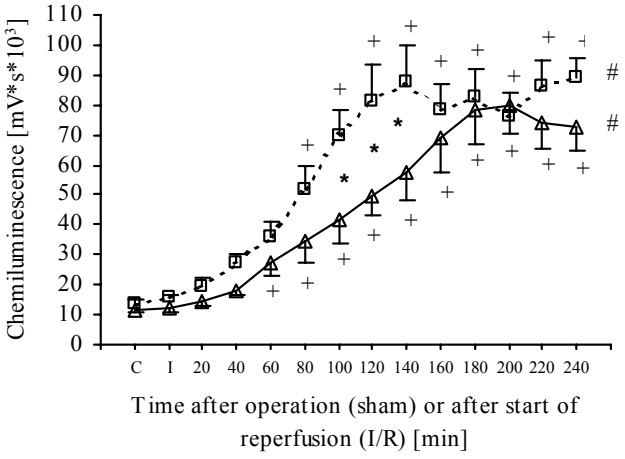

Fig. 2. *Changes in the whole blood chemiluminescence. C: preischemic control; I: end of ischemia. Solid line indicates the experimental (ischemia/reperfusion) group, dashed line indicates the sham-operated group. For other symbols and explanations see Figure 1.*

Total T cell numbers did not change. T cells consisted of 76±12 % of the total lymphocyte population and there was no significant change in this parameter in any group during reperfusion.

The relative numbers of B cells were significantly lower in both sham-operated controls (13.2±4.4 % of lymphocytes) and the experimental group (12.2±5.6 %) after two hours of reperfusion. At 4 hours of reperfusion the difference was significant only in the experimental group (7.9±4.4 %), while the decrease to 16±5.4 % in the sham-operated group was not significant. The pre-occlusion control level was 23±5.2 %.

Table 1. The relative number of the T helper cells (CD4+) and the T cytotoxic/suppressor cells (CD8+) expressed as the percentage of the total T cell population.

Time after operation (sham) or after reperfusion (I/R)			
CD8+ cells	Control	Sham	I/R
	19.0±3.0		
2 h		22.0±3.0	26.0±3.5*
4 h		22.0±3.0	24.0±2.5*
CD4+ cells	Control	Sham	I/R
	64.5±5.0		
2 h		71.0±2.5	67.5±3.0
4 h		68.0±3.5	67.5±2.0

Significant differences between I/R and control groups are indicated by asterisks.

The relative number of T helper cells (CD4+) did not change, while that of the T cytotoxic/suppressor cells (CD8+) increased significantly only in the experimental group at the second hour and the fourth hour of reperfusion (Table 1). 6.0±0.3 % of the T cells showed a CD4 and CD8 double positivity in the controls, and this number decreased to 2.1±0.1 % in the experimental group at the end of reperfusion.

Discussion

The present intestinal ischemia/reperfusion study showed that PMNL were mobilized during the second

hour of reperfusion and there was a simultaneous increase in the CL signal of the whole blood at the same time. Chemiluminescence corrected for 10^3 PMNL did not change during reperfusion. The lymphocyte number decreased mainly due to a change in the B cell counts.

In previous studies we found an increase in the PMNL and a decrease in the lymphocyte counts (Hamar *et al*. 1994, Lojek *et al*. 1997b). However, cell counts were determined every hour following reperfusion and there was a rise in PMNL counts only at the end of the third hour. The present series shows that the increase in granulocyte counts already starts at the end of the second hour of reperfusion. The granulocytosis coincides with the inflammatory cell infiltration of the damaged intestinal mucosa (Illyés and Hamar 1992).

The present protocol is similar to our earlier mesenteric shock model (Lojek *et al*. 1997b) in which we reported the same volume replacement. There was a difference between the sham-operated and experimental groups with respect to the outcome of the volume load. In the sham-operated group the supplied fluid resulted in a decrease in hematocrit, while the applied volume replacement in the experimental group prevented the hemoconcentration (Lojek *et al*. 1997b, Hamar *et al*. 1987).

The mobilization of PMNL must take place from their storage sites, probably from the bone marrow. Increased flow velocity as a mobilizing factor can be excluded since both the blood pressure and the cardiac output decreased during the second hour of reperfusion, according to our earlier experiments (Hamar *et al*. 1994). However, increased flow velocity can mobilize leukocytes in the sham-operated group, in which the volume load resulted in an increased cardiac output and a higher flow velocity in the periphery. Surgical stress and the consequent higher levels of circulating catecholamines as mobilizing factors should also be taken into consideration in both experimental and sham-operated groups.

Spontaneous CL was similar in all the samples analyzed. These results differ from those of Kadersky *et al*. (1995) who found a 2.5-fold increase of the superoxide production by the PMNL without zymosan stimulation in a similar ischemia/reperfusion experimental paradigm. However, they had a two-hour ischemia model and they analyzed the function of isolated PMNL. The isolation procedure could significantly modify the PMNL-derived ROS production.

The present model does not produce complete ischemia along the whole small intestine (Megison *et al*. 1990, Horton 1992). Collaterals can reach gut segments, which are severely hypoperfused, and the small amount of venous blood can carry signals/mediators to the general circulation during the ischemic period. These could include radicals (Weixiong *et al*. 1994), TNF-α (Grotz *et al*. 1995), arachidonic acid metabolites (Mullane *et al*. 1987) and/or other mediators able to mobilize PMNL.

There are major changes in the cellular immune response following injury (Organ *et al*. 1988, O'Sullivan *et al*. 1995, Lane *et al*. 1997). These changes of the immune system often result in immunodepression in different forms of trauma and can be expressed as variations of cell counts and as functional changes in immune cells (Xu *et al*. 1998, O'Mahony *et al*. 1985). The sympathoadrenal system is activated at very early stages of shock (Chien 1967) and the release of cortisol can elicit a decrease in lymphocyte counts (Trachte 1983). There are several compartments of the lymphoid organs that can accumulate immune cells after injury (Organ *et al*. 1988). However, it has not yet been specified which type of the T or B cell population is affected. We found an early small increase in the Tc/s (CD8+) population. The overall decline in lymphocyte counts can be explained by the change in B cells which are very likely to be attracted by the lymph nodes, sites of their origin, most likely the mesenteric lymph nodes.

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