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Human Neutrophil Mobilization during Open Heart Surgery

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Summary

Phagocyte released reactive oxygen species are often discussed in connection with ischemic and reperfusion injuries to the myocardium. The kinetics of the accumulation and oxidative burst of human blood phagocytes was studied by chemiluminescence during open heart surgery in the myocardium of human patients. Direct evidence is presented for an accumulation of neutrophils along with their markedly increased metabolic activity (oxygen radical formation), especially following the reperfusion of the ischemic myocardium. Leukocyte numbers and activity remained significantly elevated even in the venous blood obtained 24 h after the operation.

Key words
Reactive oxygen species – Chemiluminescence – Myocardium – Reperfusion

Introduction

The oxygen metabolism of cells gives rise to a number of free oxygen radicals, such as the superoxide anion, hydrogen peroxide and hydroxyl radical. In a healthy individual, these reactive oxygen species (ROS) are quickly removed to minimize any possible damages to the surrounding tissues. However, human activity of various kinds has in recent years introduced a number of toxic factors into our environment, and these, along with the lack of food or unbalanced nutrient composition, and as a result of various therapeutic procedures reduce the efficacy of body's own antioxidant adaptation mechanisms. The resulting ROS excess precipitate serious damages linked to various pathological processes, thus implicating ROS in more than 50 diseases (Halliwell 1987, Halliwell 1989).

The ways of ROS formation *in vivo* include reactions catalyzed by xanthine oxidase, autooxidation of chemically reactive substances in processes accompanying the reduction reactions of the mitochondrial electron transfer system and in several other systems. However, one of the principal sources of ROS are the phagocytic cells with neutrophils in the lead.

An increasing body of evidence has accumulated on the significance of phagocytes in the pathogenesis of a number of serious diseases (Go *et al.* 1988, Halliwell 1989). On their activation, phagocytes, the major cells of inflammation, can embolize microvascular regions, adhere to the endothelium, migrate through interendothelial cell junctions and accumulate in subcellular zones. At the same time, they damage the endothelium through the release of proteases and ROS into their surroundings (Dinerman and Mehta 1990).

This is also what occurs in connection with heart operations, tissue and organ transplantations and other surgical interventions, when ischemic organs and tissues are suddenly reperfused with oxygenated blood. Such treatments result in a number of ultrastructural, metabolic, vascular, electrophysiological and other abnormalities that are often closely linked to the activation of phagocytes (Dinerman and Mehta 1990, Bolli *et al.* 1989). Although the role of phagocytes in these processes appears to be quite evident, there still remain a number of questions to be answered. One of these problems resides in the lack of direct evidence demonstrating and explaining processes accompanying

Abbreviations: CL – chemiluminescence, PBS – phosphate-buffered saline solution, PMN – polymorphonuclear leukocytes, ROS – reactive oxygen species, S.E.M. – standard error of the mean

phagocyte activation during open heart surgery with the myocardium going through a phase of ischemia, which is followed by its reperfusion with oxygenated blood. Our investigation was designed to gain a better understanding of these phenomena by studying phagocyte activation with the use of the method of luminol-enhanced chemiluminescence.

Methods

Subjects

A total of 33 patients were included in the investigation. They were examined and operated for heart disorders at the Centre of Cardiovascular Surgery, Brno. The group consisted of 29 men and 4 women of the average age of 54±9 years. In all the cases the indication was that of ischemic heart disease, but in 5 of the patients this condition was also combined with aortic valve disease.

Surgical procedure

The patients were operated under general intravenous opiate anesthesia, under conditions of extracorporeal circulation and systemic normothermia. During the period of extracorporeal circulation, the flow was maintained at 2.3–3.0 l/min/m^2 of the body surface and the mean pressure was kept at above 60 mm Hg. Blood was oxygenated by means of a bubble oxygenator Shiley 100A. During surgery, the method of continual hypothermic cardioplegia was employed, where the cardioplegic solution (Ringer solution for the first dose: 149.1 mmol/l Na, 20.0 mmol/l K, 2.3 mmol/l Ca, 17.6 mmol/l Cl, 2.0 mmol/l HCO_3; and for following bypasses: 149.1 mmol/l Na, 4.0 mmol/l K, 2.3 mmol/l Ca, 155.6 mmol/l Cl, 2.0 mmol/l HCO_3) was being infused through aortocoronary bypasses. The initial dose of the cardioplegic solution was 1500 ml, the mean volume of the infusion in the bypasses was 1339.5±609.8 ml depending on the duration of the ischemic arrest of the heart. The temperature of the myocardium was kept at 10–15 °C.

Blood collection

Samples of blood or of a mixture of blood with the cardioplegic solution were collected during the operation from the coronary sinus in the following intervals: first on the initiation of the extracorporeal circulation, and further collections were performed at 10-minute intervals during the heart arrest period (5 collections during ischemia and 5 during reperfusion). The last sample was collected before terminating the extracorporeal circulation. The total duration of extracorporeal circulation was 94.8±19.6 min, the ischemia period lasted 52.3±7.2 min. To evaluate the patient after the operation, venous blood of each patient was examined 24 h following the surgery. The blood samples were examined for their hematocrit values, leukocyte counts and the metabolic activity of phagocytic cells immediately after the samples had been collected.

Phagocytosis assay

The metabolic activity of phagocytes was evaluated by the method of chemiluminescence modified according to the Bio-Orbit User's Manual. Equal aliquots of heparinized blood (5 U heparin/ml of blood) from each collection were tested for both spontaneous and activated phagocytosis. Aliquots of 0.1 ml of blood were pipetted into disposable polystyrene cuvettes (Clinicon) and diluted with 0.5 ml of MEM tissue culture medium, pH 7.2–7.4 (ÚSOL Prague) containig 10^{-4} M luminol (Sigma). For the measurements of spontaneous phagocytic activity only 0.1 ml of MEM medium was then added while other parallel samples received 0.1 ml of 1 % suspension of amylum oryzae (rice starch) in PBS as a particulate activator of phagocytosis.

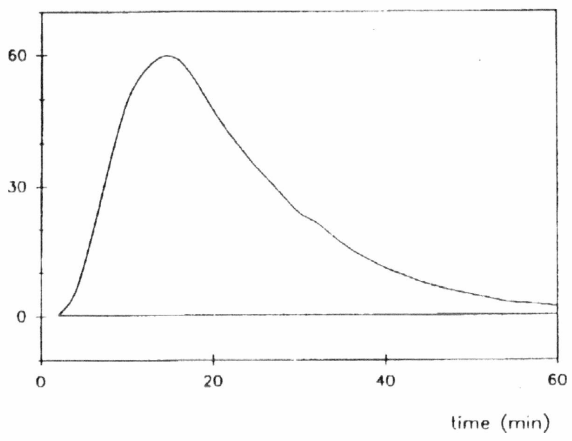

Fig. 1
The kinetics of luminol-enhanced chemiluminescence (CL) of human phagocytes in a whole blood sample activated by starch grains (full line).

In these samples the kinetics of luminol-enhanced chemiluminescence (i.e. the metabolic activity of phagocytes in the blood sample) were measured for a period of 60 min using Luminometer 1251 (Bio-Orbit, Finland) where 25 samples can be measured at a time. The values recorded during the studied time interval included the time and intensity of the peak activity (mV) and the integral area under the obtained curve which corresponds to the total amount

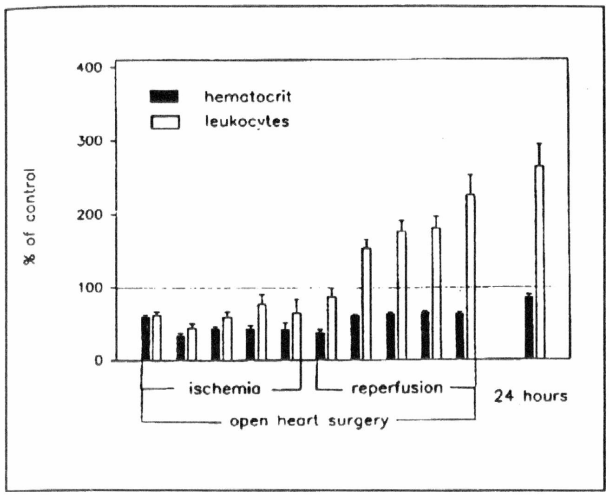

Fig. 2
Development of hematocrit values and leukocyte counts during heart ischemia and reperfusion phases of operation. Samples obtained before the operation were taken as controls (=100%).

of light produced during this selected period of time (mV/60 min). Index of phagocytosis was then calculated from these integral values of chemiluminescence as a ratio of activated to spontaneous phagocytosis. A typical curve illustrating the kinetics of phagocytosis (namely ROS production or chemiluminescence) is shown in Fig. 1.

Data analysis

The number of leukocytes, hematocrit, and values expressing the metabolic activity of phagocytes (peak, integral, index of phagocytosis) obtained for each patient from their venous blood samples collected before the start of the operation were taken as control values for that particular patient. The data recorded from testing further samples for each patient during the operation were then expressed as percentages relative to the corresponding control values. The resulting percentages were then used to calculate the arithmetical means and standard errors of the mean for all the patients included in the study.

Results

Hematocrit and the total leukocyte count

Ten minutes after the commencement of extracorporeal circulation and the introduction of the initial dose of the cardioplegic solution, the hematocrit and leukocyte count values dropped down to 33.9±3.3 and 44.1±6.1 % of the pre-operation reference values, respectively (Fig.2). In the following stages of the operation the hematocrit values fluctuated below 50 % of the control level and were increased up to 60–65 % of the controls after releasing the aortal clamp. However, even 24 h after the operation the hematocrit value was only 85±4.4 % of the control.

Leukocyte numbers presented a different picture. Within the period of reversible ischemia of the myocardium the leukocyte counts were lower than the control level since the blood was diluted with the cardioplegic solution, although in contrary to the hematocrit value, there was an apparent tendency for the leukocyte numbers to rise (Fig. 2). After releasing the aortal clamp, a steep rise in leukocytes was recorded reaching 225±27.1 % of the control before the termination of the extracorporeal circulation (probably due to the restored and activated chemotactic mechanisms).

Phagocytosis assay

As can be seen in Fig. 3, the peak and integral values as well as the index of phagocytic activity are in good correlation, and all these data reflect apparent changes in the metabolic activity (oxidative burst) of phagocytes occurring during the operation involving ischemia of the myocardium and the subsequent reperfusion with oxygenated blood. The metabolic activity of phagocytes was already rising during the period of ischemia and it exceeded the control pre-operation level at the end of this period. After releasing the aortal clamp, there was fast activation of phagocytes which reached a three-fold level compared with the control before the termination of the extracorporeal circulation (305.1±21.4 % of peak activity). A three-fold increase in phagocyte activity was also recorded 24 h after the operation (331.8±47.8 %), but it should be pointed out that this last value was obtained from samples of peripheral blood of the patients, so that even higher phagocyte activity and thus ROS formation can be expected within the myocardium itself at this time.

In order to evaluate the relationship between the increased numbers of leukocytes and their activity during the operation we plotted the two variables on the same graph (Fig. 4). This comparison reveals that both factors apparently participate in the increased ROS production, i.e. increased accumulation of phagocytes occurs within the myocardium due to the activation of chemotactic mechanisms, and in addition, these phagocytes are strongly stimulated to a higher production of reactive oxygen species.

Discussion

There is a growing body of evidence for a number of changes associated with the reperfusion of ischemic organs. Such events include a calcium overload within the cells, an influx of inflammatory cells into the affected areas, generation of oxygen-

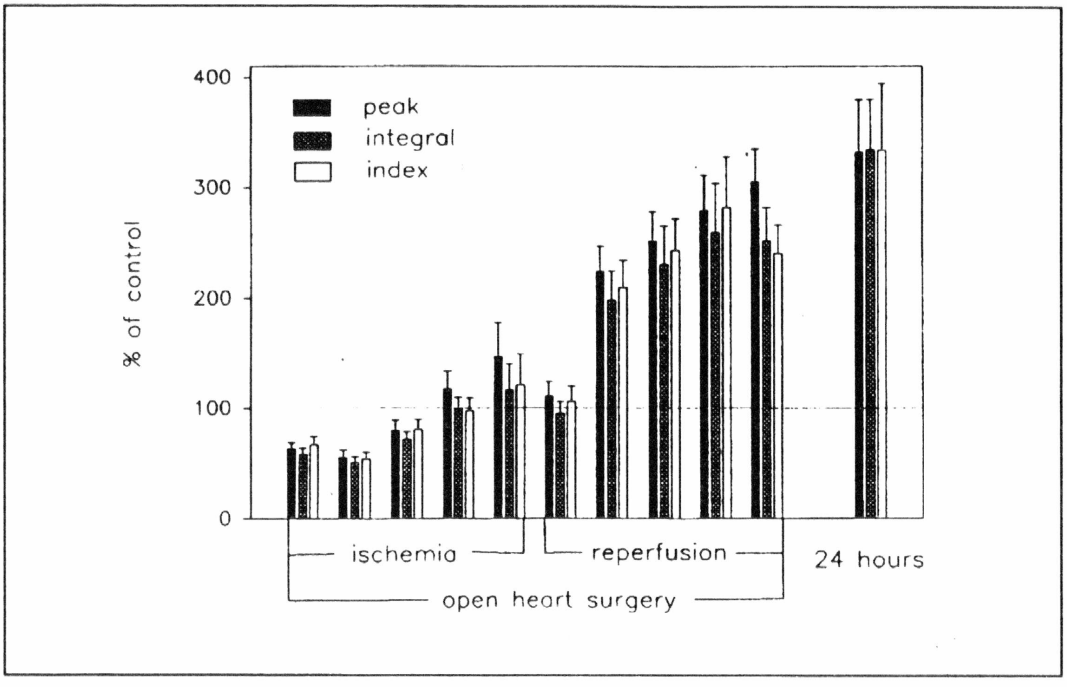

Fig. 3
Values of chemiluminescence (peak activity, integral) and index of phagocytosis (see text) recorded for samples collected during operation (phases of ischemia and reperfusion) and 24 h after the operation, expressed as percentages of control samples obtained before the operation.

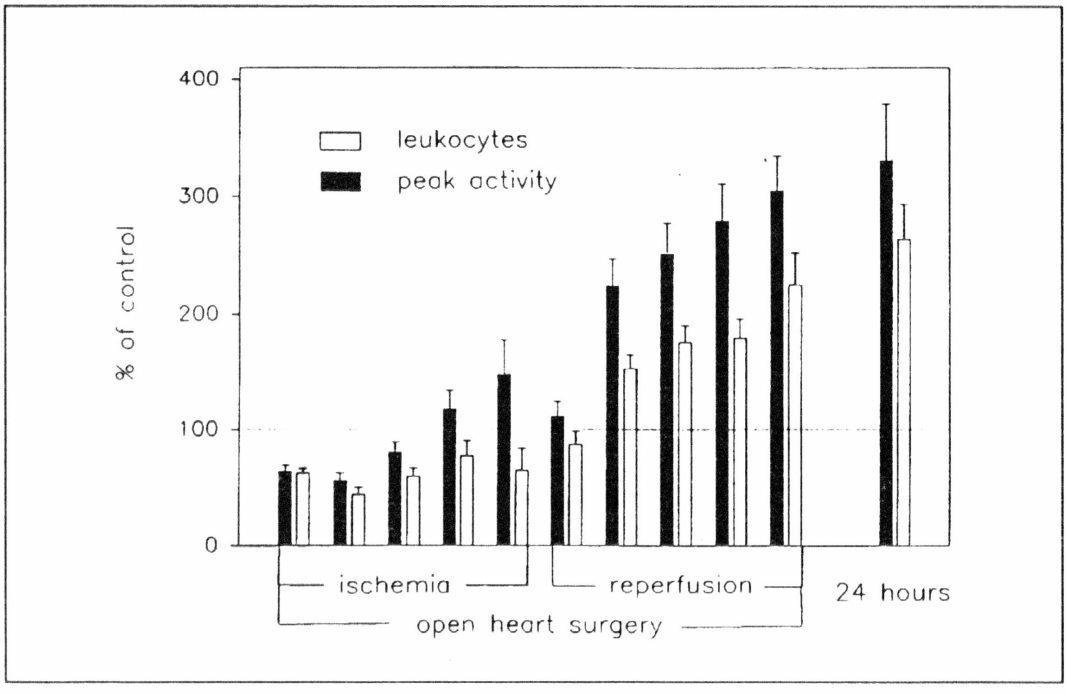

Fig. 4
Changes in leukocyte numbers and peak values of luminol-enhanced chemiluminescence (oxidative burst) in blood samples obtained during phases of heart ischemia, reperfusion with oxygenated blood and 24 h after the operation. Values are expressed as percentages of control samples obtained before the operation.

derived free radicals and a release of proteolytic enzymes accompanying phagocyte activation (Lucchesi 1990). Excess production of free radicals affects the metabolism of the cells exposed to these agents and causes damages to viable tissues *via* lipid peroxidation and the oxidation of protein sulfhydryl groups, which leads to the perturbation of membrane permeability and enzyme function. Free radicals are also known to interfere with nucleic acids, cytosolic molecules and the components of the extracellular matrix (Halliwell and Gutteridge 1989, Lucchesi 1990, Werns and Lucchesi 1990, Chi *et al.* 1989).

In our work, we investigated the metabolic activity of PMN leukocytes obtained directly from the human ischemic myocardium during surgical treatment of ischemic heart disease. We found the metabolic activity of the PMNs rising already during the period of surgery-induced ischemia. This finding is in agreement with the results reported in some experimental studies involving dogs, where PMN activity rose some 15–20 min after occluding a major coronary artery (Mullane *et al.* 1987). An increased accumulation of neutrophils within the ischemic myocardium even before its reperfusion was also reported in earlier work studying myeloperoxidase activity after coronary artery occlusion (Mullane *et al.* 1985), or in studies involving the use of ^{111}In-labelled neutrophils (Go *et al.* 1988).

One of the possible explanations can be seen in the activation of complement proteins during the ischemic period, resulting in PMN migration into the myocardium (O'Neill *et al.* 1989). After migrating into the affected region, neutrophils discharge ROS, proteolytic enzymes and arachidonic acid metabolites, such as leukotriene B_4 with powerful chemotactic effects, which encourage further migration of PMNs into the myocardium.

In our study, the reperfusion of the human ischemic myocardium in the course of open heart surgery was accompanied by a significant elevation in the number of leukocytes and more than a three-fold increase in their chemiluminescence attesting to an accelerated recruitment of PMNs in this phase of the operation.

A sudden rise in ROS production within the hearts of experimental dogs associated with reperfusion was also reported by Bolli and co-workers (Bolli 1988, Bolli *et al.* 1988). The authors measured ROS production *in situ*, using spin traps and electron paramagnetic resonance spectroscopy. In their study, the myocardial production of oxygen radicals already began during coronary occlusion, but increased dramatically within the first few minutes after reperfusion, peaking (approximately 100-fold above ischemic levels) 2-4 min after the reflow. After this initial burst, the production of radicals abated, but did not cease, persisting up to 3 h of the follow-up period. However, one can speculate that these ROS measured by Bolli and co-workers originated not only from neutrophil activity, but an appreciable role should also be ascribed to xanthine oxidase produced in significant amounts during ischemia by the endothelial cells of the canine heart.

The contribution of xanthine oxidase to ROS production in the heart tissue of man has been a matter of controversy (Bolli 1988) and our study could not elucidate this issue. Our monitoring of the neutrophil activity did, however, reveal a marked activation of these phagocytic elements within the human myocardium following reperfusion, with ROS production rising constantly over the whole period of operation.

A considerable elevation in the phagocyte activity was also observed in venous blood samples obtained from our patients 24 h after their operation. This result is also consistent with the finding of Prasad *et al.* 1989, who measured chemiluminescence of PMNs isolated from mixed venous blood samples obtained from a group of dogs with chronic mitral insufficiency. They observed a four-fold level of PMN activity compared with the healthy control mongrel dogs.

It is rather important to study the accumulation and metabolic activity of neutrophils in connection with ischemia and reperfusion of the myocardium, as excessive activation of phagocytes is high on the list of undesiderable side effects accompanying surgical treatment of ischemic heart disease and threatening the patient with the subsequent development of reocclusion. Some 10–20 % patients develop reocclusions within 6-12 months following coronary bypass grafting (Dinerman and Mehta 1990).

The problem of neutrophil recruitment and activation during heart surgery has been tackled by a number of investigators. Some strategies involve attempts to eliminate neutrophils from the circulation by administering goat antiserum against PMN (O'Neill *et al.* 1989), or to inhibit the neutrophils by using a monoclonal antibody against Mo-1 leukocyte integrin, i.e. the CD11b/CD18 cell adhesion molecule (Simpson *et al.* 1988, 1990). These protocols have offered some approaches to solving the ischemia-reperfusion associated problems, but still more studies are needed to evaluate the use of various inhibitors of the oxidative burst of activated neutrophils, such as cyclooxygenase and lipoxygenase inhibitors in the metabolism of eicosanoids, calcium blockers, or the application of ROS scavengers, such as superoxide dismutase and catalase (Mullane *et al.* 1987, Janero *et al.* 1988, Werns *et al.* 1988, Mehta *et al.* 1989, Tamura *et al.* 1988).

However, in the above mentioned studies animals were used as experimental subjects, while not enough data have been available on the peri-operation kinetics of neutrophil mobilization in human patients.

This present work shows that the method of luminol-enhanced chemiluminescence can successfully be used in such studies.

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References

BOLLI R.: Oxygen-derived free radicals and postischemic myocardial dysfunction ("stunned myocardium"). *J. Am. Coll. Cardiol.* **12:** 239–249, 1988.

BOLLI R., PATEL B.S., JEROUDI M.O., LAI E.K., McCAY P.B.: Demonstration of free radical generation in "stunned" myocardium of intact dogs with the use of the spin trap α-phenyl-N-tert-butyl nitrone. *J. Clin. Invest.* **82:** 476–485, 1988.

BOLLI R., JEROUDI M.O., PATEL B.S., ARUOMA O.I., HALLIWELL B., LAI E.K., McCAY P.B.: Marked Reduction of Free Radical Generation and Contractile Dysfunction by Antioxidant Therapy Begun at the Time of Reperfusion. *Circ. Res.* **65:** 607–622, 1989.

DINERMAN J.L., MEHTA J.L.: Endothelial, Platelet and Leukocyte Interactions in Ischemic Heart Disease: Insights Into Potential Mechanisms and Their Clinical Relevance. *J. Am. Coll. Cardiol.* **16:** 207–222, 1990.

GO L.O., MURRY C.E., RICHARD V.J., WEISCHEDEL G.R., JENNINGS R.B., REIMER K.A.: Myocardial neutrophil accumulation during reperfusion and reversible ischemic injury. *Am. J. Physiol.* **255:** H1188–H1198, 1988.

HALLIWELL B.: Oxidants and human disease: some new concepts. *FASEB J.* **1:** 358–364, 1987.

HALLIWELL B.: Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. *Br. J. Exp. Path* **70:** 737–757, 1989.

HALLIWELL B., GUTTERIDGE J.M.C.: Free Radicals in Biology and Medicine. Clarendon Press, Oxford, 1989.

CHI L., TAMURA Y., HOFF P.T., MACHA M., GALLAGHER K.P., SCHORK M.A., LUCCHESI B.R.: Effect of superoxide dismutase on myocardial infarct size in the canine heart after 6 hours of regional ischemia and reperfusion: a demonstration of myocardial salvage. *Circ. Res.* **64:** 665–675, 1989.

JANERO D.R., BURGHARDT B., LOPEZ R.: Protection of cardiac membrane phospholipid against oxidative injury by calcium antagonists. *Biochem. Pharmacol.* **37:** 4197–4203, 1988.

LUCCHESI B.R.: Modulation of leukocyte-mediated myocardial reperfusion injury. *Annu. Rev. Physiol.* 52: 561–576, 1990.

MEHTA J.L., NICHOLS W.W., DONNELLY W.H., LAWSON D.L., THOMPSON L., RIET M., SALDEEN T.G.P.: Protection by superoxide dismutase from myocardial dysfunction and attenuation of vasodilator reserve after coronary occlusion and reperfusion in dog. *Circ. Res.* **65:** 1283–1295, 1989.

MULLANE K.M., KRAEMER R., SMITH B.: Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. *J. Pharmacol. Methods.* **14:** 157–167, 1985.

MULLANE K.M., SALMON J.A., KRAEMER R.: Leukocyte-derived metabolites of arachidonic acid in ischemia-induced myocardial injury. *Federation Proc.* **46:** 2422–2433, 1987.

O'NEILL P.G., CHARLAT M.L., MICHAEL L.H., ROBERTS R., BOLLI R.: Influence of neutrophil depletion on myocardial function and flow after reversible ischemia. *Am. J. Physiol.* **256:** H341–H351, 1989.

PRASAD K., KALRA J., CHAN W.P., CHAUDHARY A.K.: Effect of oxygen free radicals on cardiovascular function at organ and cellular levels. *Am. Heart J.* **117:** 1196–1202, 1989.

SIMPSON P.J., TODD R.F., FANTONE J.C., MICKELSON J.K., GRIFFIN J.D., LUCCHESI B.R.: Reduction of experimental canine myocardial reperfusion injury by a monoclonal antibody (Anti-Mo1, Anti-CD11b) that inhibits leukocyte adhesion. *J. Clin. Invest.* **81:** 624–629, 1988.

SIMPSOM P.J., TODD R.F., MICKELSON J.K., FANTONE J.C., GALLAGHER K.P., LEE K.A., TAMURA Y., CRONIN M., LUCCHESI B.R.: Sustained limitation of myocardial reperfusion injury by a monoclonal antibody that alters leukocyte function. *Circulation* **81:** 226–237, 1990.

TAMURA Y., CHI L., DRISCOLL E.M. JR., HOFF P.T., FREEMAN B.A., GALLAGHER K.P., LUCCHESI B.R.: Superoxide dismutase conjugated to polyethylene glycol provides sustained protection against myocardial ischemia/reperfusion injury in canine heart. *Circ. Res.* **63:** 944–959, 1988.

WERNS S.W., SIMPSON P.J., MICKELSON J.K., SHEA M.J., PITT B., LUCCHESI B.R.: Sustained limitation by superoxide dismutase of canine myocardial injury due to regional ischemia followed by reperfusion. *J. Cardiovasc. Pharmacol.* **11:** 34–44, 1988.

WERNS S.W., LUCCHESI B.R.: Free radicals and ischemic tissue injury. *TiPS.* **11:** 161–166, 1990.

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