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Flow Cytometric Analysis of Peroxidative Activity in Granulocytes From Coronary and Peripheral Blood in Acute Myocardial Ischemia and Reperfusion in Dogs: Protective Effect of Methionine

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Background: Methionine has shown protective effects in experimental models of myocardial infarction and is highly reactive to oxidative compounds produced by polymorphonuclear leukocytes (PMN), which in turn have been associated with myocardial damage. We have investigated the effect of methionine administration on spontaneous leukocyte peroxidative activity in myocardial ischemia and reperfusion.

Methods: In anesthetized dogs, with coronary occlusion (90 min) and reperfusion (90 min), PMN activation was measured by flow cytometric determination of H_2O_2 with dihydrorhodamine 123, and correlated to hemodynamic parameters and infarct presence. To assess a possible direct effect of methionine, H_2O_2 and superoxide were measured by flow cytometry in dog leukocyte suspensions following in vitro stimulation with f-MLP.

Results: PMN peroxidative activity in saline-treated dogs increased significantly after coronary occlusion and after reperfusion. These changes were greater in coronary venous blood than in femoral blood. Methionine administration (150 mg/kg, i.v.) before occlusion totally suppressed PMN activation, both after occlusion and reperfusion.

Conclusions: PMN are promptly activated in myocardial ischemia, and methionine administration prevents such activation. However, methionine has no direct effect on spontaneous peroxidative activity, and f-MLP induced peroxidative activity. These in vivo effects of methionine, may additionally contribute to explain its protective role in experimental myocardial ischemia. Cytometry 37:140–146, 1999. © 1999 Wiley-Liss, Inc.

Key terms: myocardial infarction; granulocytes; peroxidative activity; methionine; flow cytometry

Myocardial ischemia and reperfusion is accompanied by increased local production of toxic reactive oxygen (ROS) and nitrogen (RNS) species and loss of protective antioxidant mechanisms (1,2), as well as by leukocyte entrapping in the surrounding area (3,4). These circumstances have been shown to be deleterious for the myocardium (4–6). Several studies have demonstrated that neutrophil depletion, and/or administration of drugs that inhibit leukocyte infiltration protects myocardial tissue in animal models of ischemia and reperfusion (4,6,7). In this regard, it has been reported that methionine, a sulfur-containing amino acid, exhibits beneficial effects in experimental models of myocardial ischemia (8–11), as well as a high reactivity against oxidative compounds produced and released by granulocytes, such as chloramines and hypochlorous acid (12,13), that are involved in myocardial ischemic damage (6,14,15).

The investigation of the role of granulocytes in the tissue injury that follows myocardial ischemia and reperfusion requires methods that are suitable to assess early changes in oxidative activity of leukocytes localized close to the damaged area. On one hand, it is a relatively common clinical practice to guide catheters into the coronary veins of patients suffering from several types of heart conditions, thus allowing easy sampling of local blood cells. On the other hand, flow cytometry is a sensitive analytical ap-

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proach to quantitate the spontaneous or stimulus-induced generation of ROS in a wide variety of clinical and experimental conditions (16–18).

Dihydrorhodamine 123 (DHRH123) is the nonfluorescent reduced form of rhodamine 123 (RH123), a mitochondrion-selective probe with a strong green fluorescence (19). DHRH123 is taken up readily by leukocytes, and upon oxidative burst is oxidized in the presence of myeloperoxidase to yield RH123 (17). As the increase of green fluorescence is quantitatively related to the intensity of oxidative burst, the system DHRH123/RH123 has been repeatedly used for flow (17,20,21,22) and image (23) cytometry analysis of leukocyte oxidative activity.

Therefore, we have applied flow cytometry to quantitate the oxidative response of granulocytes in the course of a cycle of acute coronary occlusion and reperfusion in dogs, as well as to assess the possible protective effects of methionine administration over that peroxidative activity. Our results show that flow cytometry is suitable to detect rapidly and to follow functional changes in local granulocytes during ischemic injury, as evidenced by an increased intracellular oxidative activity, which is prevented when dogs are pretreated with methionine.

MATERIALS AND METHODS

Chemicals

L-Methionine was obtained from Merck (Darmstadt, Germany). RPMI culture medium, dimethyl sulfoxide (DMSO), Ficoll Histopaque (density 1.077), *N*-formyl-methionyl-leucyl-phenylalanine (f-MLP) and nitro blue tetrazolium (NBT) were from Sigma Chemical Co. (St. Louis, MO). Dihydrorhodamine 123 (DHRH 123), hydroethidine (HE) and propidium iodide (PI) were from Molecular Probes (Eugene, OR).

Surgical Preparation and Experimental Protocol

All the procedures were performed in accordance with the agreements of the European Convention of Strasbourg of March 18, 1986. Eight healthy adult mongrel dogs (15–25 kg) with food intake restricted overnight and free access to water were separated randomly in two groups of four (control group and L-methionine group). Animals were anesthetized with 25 mg/kg i.v. sodium thiopental (Pentothal, Abbott), and given additional doses throughout the experiment when necessary. Dogs were intubated and ventilated with room air by means of a respirator, and blood gases were determined at intervals to assure adequate ventilation. The mean arterial blood pressure (MAP) was measured via a catheter inserted into the femoral artery, placed at the aortic origin and attached to a Gould Statham P23ID transducer. MAP, heart rate (HR) and EKG were recorded with an Elema Schonander Mingograf (Siemens) polygraph. The femoral vein was catheterized for saline and methionine administration.

Thoracotomy was performed at the left fifth intercostal space, and the heart was suspended in a pericardial cradle. At this time, dogs received an i.v. injection of saline or L-methionine (150 mg/kg body weight), respectively, 5 min prior to coronary occlusion, which was achieved by ligation of the left anterior descending coronary artery at a point just distal to its first major diagonal branch. The coronary artery was occluded for 90 min, and after ligation withdrawal the myocardium was reperfused for 90 min. After reperfusion, the heart was excised and the left ventricle cut into 1-cm thick transverse slices that were incubated with nitro blue tetrazolium (NBT) solution, that consisted of Sorensen's phosphate buffer (0.1 M) pH 7.4 and 0.5 per ml of NBT (24).

Ex Vivo Analysis of H_2O_2 Generation by Granulocytes

Whole blood samples (1 ml) were obtained from the femoral and local coronary veins of control or L-methionine-treated dogs immediately before the coronary occlusion; 10 and 90 min after occlusion; and 5 and 90 min after reperfusion. In addition, one sample of saphenous vein blood was taken immediately before anesthesia administration to establish the basal H_2O_2 intracellular content. Leukocytes were isolated from EDTA-anticoagulated blood by sedimentation in 300 μl of 6% dextran, and contaminating erythrocytes were removed by hypotonic lysis with 0.83% NH_4Cl followed by centrifugation.

Intracellular generation of H_2O_2 was estimated with the fluorochrome DHRH123 (17,20–23). Cells were resuspended (≈250,000 cells/ml) in RPMI medium and incubated with 5 μM DHRH123 (from a 1 mM stock solution in DMSO) for 15 min at 37°C in the dark. Immediately prior to flow cytometric analysis, cell suspensions were transferred to an ice bath and incubated for another 5 min with 5 μg PI/ml (from a 1 mg/ml stock solution in PBS) in order to exclude dead cells.

In Vitro Analysis of L-Methionine Effect on Intracellular H_2O_2 and Superoxide Ion Generation in Granulocytes From Normal Dogs

Whole blood samples (3 ml) from the saphenous vein of a separate group of untreated dogs were anticoagulated with EDTA and leukocytes were purified by sedimentation on Ficoll, as described by Robinson et al. (16). Briefly, 300 μl of blood were carefully layered on the top of 500 μl Ficoll Histopaque (density 1.077) in an Eppendorf tube and left undisturbed in a stable tube rack for 20–30 min at room temperature. Under these conditions, erythrocytes aggregate and precipitate spontaneously through the Ficoll, while the top layer is progressively enriched in white blood cells and platelets. When the plasma layer appears clear from erythrocytes, the upper 200 μl are carefully withdrawn and resuspended in warm RPMI medium (≈250,000 cells/ml) in the presence of 0–1mM L-methionine for 30 min.

Basal or f-MLP-induced generation of H_2O_2 and $O_2^{\cdot -}$ was estimated simultaneously with DHRH123 and HE, respectively. Control- or L-methionine-treated cell suspensions were incubated with 5 μM DHRH123 and 5 μM HE (both from 1 mM stock solutions in DMSO) for 15 min at 37°C in the dark. Cell activation was induced by the addition of 2 μM f-MLP for 10 min and then stopped by transferring

cell suspensions to an ice bath prior to flow cytometric analysis.

Flow Cytometry

Analysis was performed using an EPICS Elite cell sorter or an EPICS XL-MCL (Coulter Electronics, Hialeah, FL) both equipped with a 488 nm argon laser (Cyonics, San Jose, CA). Forward-angle light scatter (FALS) and right-angle light scatter (90LS), as well as negative PI-staining, were used to discriminate between viable and nonviable granulocytes. Fluorescence was collected through a 488-nm blocking filter, a 525-nm bandpass for DHRH 123 and a 575-nm bandpass for HE or PI. Correlated histograms and listmode data were collected for linearly (FALS, 90LS) or logarithmically amplified signals (DHRH 123, HE and PI), and the intracellular H_2O_2 and $O_2^{\cdot -}$ content were estimated as fluorescence arbitrary units.

In our experimental procedure for in vitro flow cytometric assays, the blood cell suspensions obtained from control and experimental dogs were comprised of mononuclear cells and granulocytes. Granulocytes were easily distinguishable in the flow cytometer by their higher forward and side scatter signals, while monocytes and lymphocytes were not clearly separated from each other (Fig. 1A). In order to include only granulocytes in the fluorescence measurements, a discriminator threshold was set up on forward scatter below the high forward scatter population, thus excluding mononuclear cells, dead cells, and debris (residual platelets and erythrocytes). The adequate selection of the discriminator was confirmed by control experiments of PMA-activation (1 μM for 15 min at 37°C) of leukocytes stained with DHRH123, which showed both that isolated granulocytes from coronary and femoral veins were responsive to external activation stimuli and that almost all the granulocytes over the threshold were recovered as highly fluorescent cells (Fig. 1B).

Statistical Analysis

Hemodynamic variables and granulocyte intracellular H_2O_2 and $O_2^{\cdot -}$ levels over time within each group, and differences between groups were analyzed using analysis of variance (ANOVA) with the Tukey post-hoc test. Data are given as mean ± SD. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Hemodynamics

No significant differences in heart rate between the saline- and methionine-treated groups were observed. In addition, heart rate did no change significantly in both groups of dogs throughout the experiment. Regarding MAP, no significant change was observed throughout the experiment in the saline and in methionine-treated group. Results are shown in Table 1.

Presence of Infarct Areas

Staining with NBT macroscopically showed the presence of myocardial infarct areas in all dogs, from the control and treated groups.

In Vivo Effect of Methionine on Intracellular Generation of H_2O_2 in Granulocytes From Local and Peripheral Blood

Figure 1 shows the morphological and fluorescence features of leukocytes isolated from the coronary vein, i.e., close to the injured myocardial area, and from a peripheral vein, i.e., distal to the ischemic and reperfused tissue. The peroxidative activity in both types of granulocytes is estimated from the DHRH123 fluorescence and expressed as the percentage of the baseline activity, determined in granulocytes obtained from the saphenous vein before anesthesia. Peroxidative activity in granulocytes isolated from peripheral (femoral) venous blood and in local (coronary) venous blood is shown quantitatively in Figure 2. In the saline-treated group, the peroxidative activity in granulocytes isolated from coronary venous blood, increased significantly following coronary artery ligation (37% and 67% after 10 and 90 min after ligation, respectively), and was further enhanced rapidly after reperfusion (145% and 180%, 5 and 90 min after reperfusion, respectively). The effect of coronary ligation on granulocyte peroxidative activity could be detected also in femoral venous blood, but with a significant delay. Thus, peroxidative activity increased significantly (44%) only 90 min after coronary occlusion. As observed in granulocytes from the coronary vein, there was an additional increase in peroxidative activity in peripheral granulocytes following reperfusion, which was of rapid appearance, but of lower intensity (73% and 80% 5 and 90 min after reperfusion, respectively, with respect to the pre-occlusion value).

Within the methionine-treated group, no significant modification of PMN peroxidative activity was observed throughout the experiment, in saphenous venous blood or in local venous blood. However, the PMN activity measured in local venous blood was significantly lower 10 and 90 min after coronary occlusion (39% and 58%, respectively), and 5 and 90 min after reperfusion (65% and 70%, respectively) in the methionine-treated group than in saline-treated group. When the measurements were performed in femoral venous blood from methionine-treated dogs, PMN activity was significantly lower before occlusion (30%), and at 10 and 90 min after coronary occlusion (37% and 48%, respectively), and at 5 and 90 min after reperfusion (64% and 65%, respectively) than in the saline-treated dogs.

In Vitro Effect of Methionine on Granulocyte Peroxidative Activity

The above results could indicate either that L-methionine might prevent directly the generation of oxidative species by leukocytes activated eventually or that this amino acid might reduce the presence or block the action of leukocyte-activating substances released during the

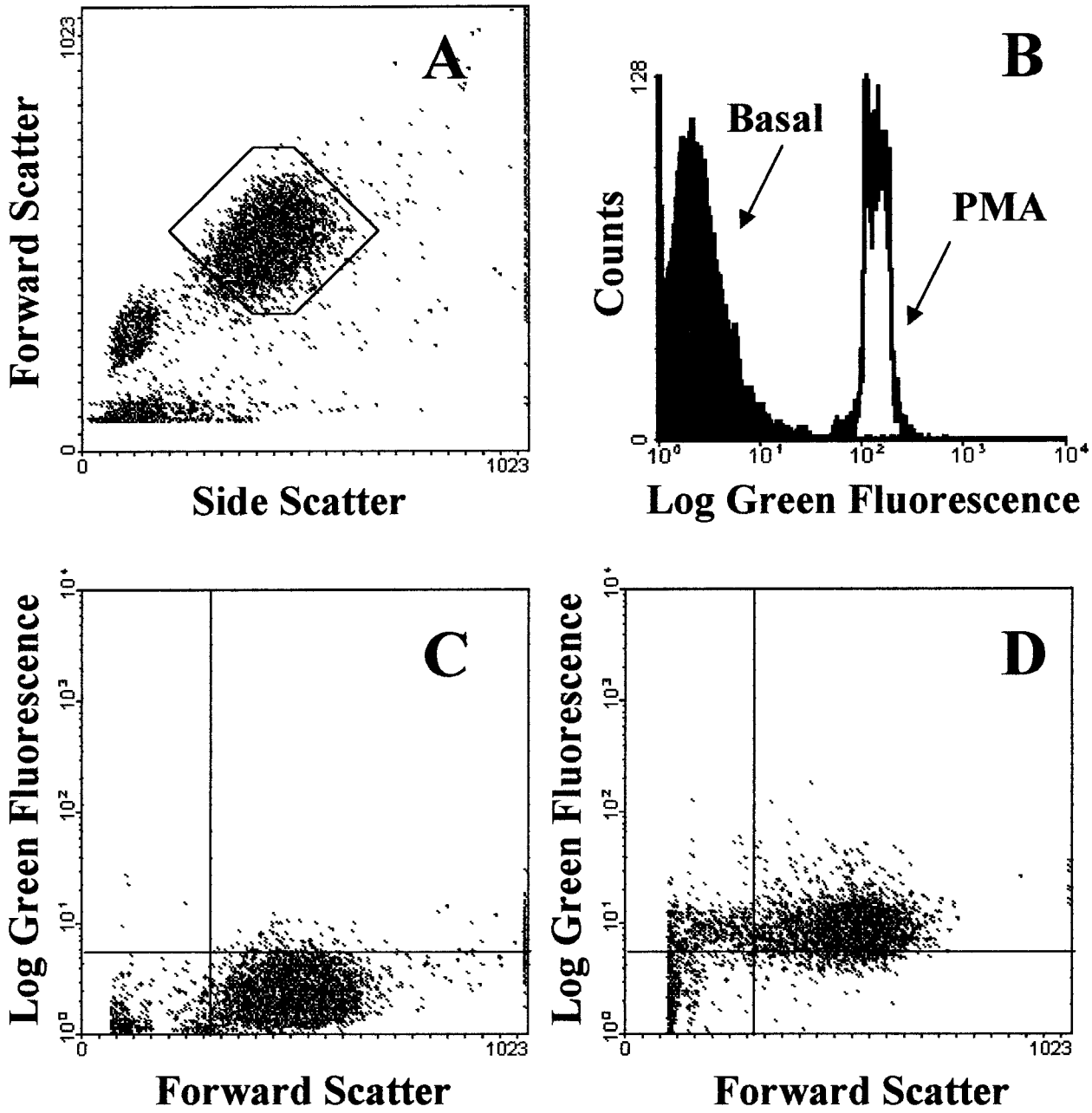

FIG. 1. Flow cytometric analysis of peroxidative activity in granulocytes isolated from the coronary vein of dogs. **A:** Light scatter properties of isolated leukocytes. The population of higher forward- and side-scatter corresponding to live granulocytes was gated for further fluorescence analysis. **B:** Overlay of green fluorescence histograms (RH123 generation from DHRH123) from light-scatter gated granulocytes in basal conditions and after incubation with PMA (1 μM, 15 min), showing that coronary vein granulocytes are responsive to an activation stimulus. **C** and **D:** Representative dot-plots of green fluorescence versus forward scatter of leukocytes isolated prior to coronary artery ligation (C) and 90 min after reperfusion (D) showing increased peroxidative activity in granulocytes. Similar morphological and fluorescence features were observed in leukocytes isolated from the femoral vein.

ischemia-reperfusion cycle (25). The first hypothesis is not supported by an earlier report by Prasad et al. (26), which showed that oxidation-related chemiluminescence of zymosan-challenged normal dog granulocytes was not affected in vitro by L-methionine.

In view of this, we decided to assess the in vitro effect of L-methionine on the generation of oxidative species (H_2O_2 and $O_2^{\cdot -}$) in granulocytes stimulated with a more powerful agonist, as is the chemotactic peptide f-MLP. Consistent with the previously published data (26), in vitro incubation with L-methionine did not significantly affect the f-MLP-triggered intracellular generation of H_2O_2 or $O_2^{\cdot -}$ in control granulocytes (data not shown). The lack of in vitro action suggests that the beneficial effects of L-methionine

Table 1
*Mean and Standard Deviation of Heart Rate (HR) and Mean Arterial Pressure (MAP) Expressed as Beats per Minute and mm Hg, Respectively**

	Pre-occlusion		Post-occlusion		Reperfusion	
	Pretreatment	Post-treatment	10 min	90 min	5 min	90 min
Saline n = 4						
HR	156 ± 32	156 ± 32	154 ± 28	159 ± 30	156 ± 22	157 ± 25
MAP	129 ± 50	129 ± 50	113 ± 39	107 ± 35	101 ± 30	101 ± 18
Methionine n = 4						
HR	154 ± 38	148 ± 35	150 ± 41	139 ± 28	146 ± 29	140 ± 15
MAP	115 ± 29	116 ± 31	113 ± 25	85 ± 45	72 ± 36	87 ± 41

*n = number of experiments.

on local and peripheral granulocytes during myocardial ischemia and reperfusion are not resulting from a direct effect of the amino acid on these blood cells.

DISCUSSION

Several studies in animal and human models indicate that granulocytes are involved in the pathogenesis of acute myocardial ischemia and the extension of myocardial injury (4,6,11,7). In the present study, we have evaluated the leukocyte peroxidative activity before and at different time periods following coronary occlusion and after reperfusion, as well as the effect of methionine administration on that activity. The use of flow cytometric techniques based upon pro-oxidant-sensitive fluorogenic substrates has allowed us to quantitate the ex vivo production by granulocytes of oxygen-related species in response to ischemia and reperfusion. The combination of in situ blood sampling techniques and flow cytometry has allowed us to determine the rapid onset of peroxidative activation in granulocytes obtained from a region closely involved in the ischemic phenomenon, consistent with previous data obtained in similar experimental models, using different techniques (26,27). Our results confirm that granulocyte activation is more prominent during the reperfusion, as shown previously (26).

The flow cytometric analysis of granulocytes from peripheral blood has provided information about the time course and duration of the changes in granulocyte activity and their possible implication in alterations occurring outside of the ischemic myocardium. Our results show that, although delayed and with lower intensity than in the coronary vein, granulocytes in peripheral blood become pro-oxidative following myocardial ischemia and reperfusion. Multiple experimental data support the theory that intracellular generation of green-fluorescent RH123 from DHRH123 is mostly dependent on H_2O_2. Thus, DHRH123 in cell-free assays does not react directly with superoxide (28) but reacts rapidly with H_2O_2 in the presence of peroxidase, cytochrome C or Fe^{2+} (28). While H_2O_2/peroxidase lead to a rapid oxidation of DHRH123, H_2O_2 alone in cell-free assays slowly oxidizes the probe (29), indicating that although necessary, H_2O_2 is not sufficient to produce a fast generation of RH123 from DHRH123. The strong catalytic effect of iron compounds on DHRH123 intracellular oxidation (29) suggests that hydroxyl radical, generated from H_2O_2 by the Fenton reaction, is essentially involved in the last steps of RH123 generation, as further supported by the inhibitory effect of iron-chelating molecules (30). The importance of H_2O_2 in DHRH123 is also stressed by the changes in DHRH123-generated fluorescence in the presence of scavengers such as catalase or when the activity of H_2O_2-consuming enzymes is inhibited (21,23).

It has been reported that DHRH123 can also be used to detect peroxynitrite, the oxidant generated by reaction of nitric oxide (NO) with superoxide or H_2O_2 (30). However, the relevance of NO or peroxynitrite production by human resting or activated granulocytes is not clear. Thus, Carreras et al. (32) showed that NO production is part of the integrated response of PMA-stimulated human PMN and suggested (but not actually proved) that the kinetics of NO and superoxide release would favor the formation of peroxynitrite. On the contrary, McBride and Brown (33) showed no significant generation of NO in resting or PMA-activated PMN, although activated cells rapidly break down exogenous NO. In this context, it is important to underline that a recent report shows that peroxynitrite, in experimental conditions leading to DHRH123 oxidation, generates H_2O_2 by reaction with HEPES (a very common buffer in cell studies) and related tertiary amines, raising doubts about the ultimate oxidant involved in peroxynitrite-dependent DHRH123 oxidation (34). In view of the above considerations, it is likely that in our experimental conditions DHRH123 is revealing essentially H_2O_2 generation by granulocytes, as assayed by flow cytometry.

L-Methionine administration, not only provides high reactivity against chloramines and HOCl, as previously described (12), but also decreases granulocyte peroxidative activity after coronary occlusion and reperfusion, both in local vein blood and in the periphery (Fig. 2). Although we have not experimentally addressed the mechanism by which methionine may inhibit leukocyte activation in our model, the lack of effect of this amino acid on the H_2O_2 and $O_2^{\cdot -}$ generation by fMLP-activated dog granulocytes seems to exclude a direct effect on intracellular peroxidative pathways. This is consistent with the earlier report by Prasad et al. (21), which showed that L-methionine did not reduce the production of prooxidant species in granulocytes during phagocytosis of zymosan particles. On the other hand, it is unlikely that L-methionine acts as a

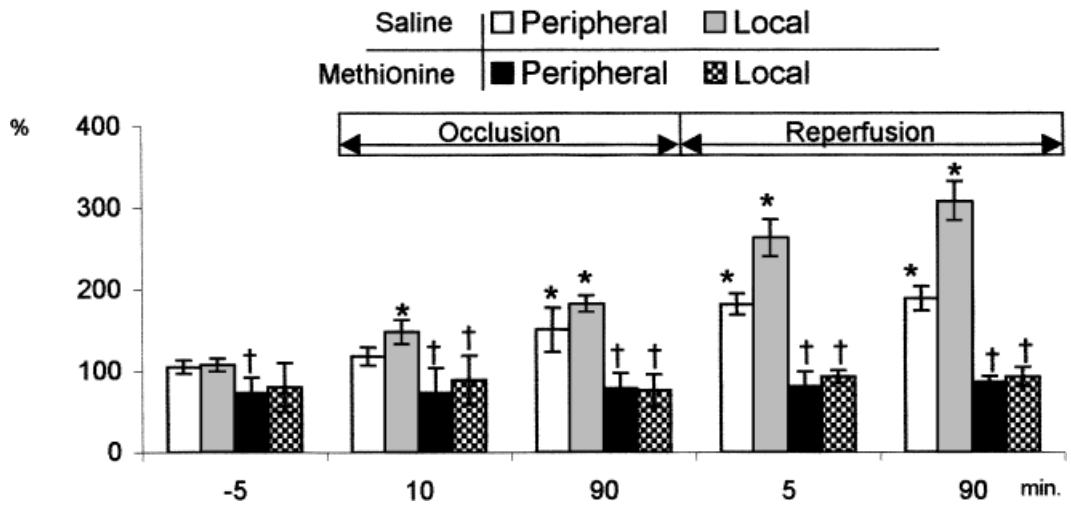

FIG. 2. Flow cytometric quantitation of the protective effect of L-methionine on the oxidative activity of granulocytes isolated from the femoral vein (peripheral) and coronary vein (local) of dogs subjected to acute myocardial ischemia and reperfusion. Data show the changes in mean fluorescence intensity of the whole population of DHRH123-stained granulocytes in each condition and are expressed as the percentage of change of mean fluorescence intensity of peripheral granulocytes obtained before anesthesia, which is considered to be 100%. Samples were obtained before coronary ligation (−5 min) and 10 and 90 min after coronary ligation (occlusion) and 5 and 90 min following reperfusion. $^*P < 0.05$ versus pre-occlusion value within each experimental condition; † $P < 0.05$ versus saline-treated group at same time point of the experiment.

scavenger of such oxygen-reactive species under our experimental conditions. However, L-methionine is known to react with other deleterious species, such as hypochlorous acid (HOCl), which are produced by activated granulocytes (12,13,35). HOCl quenching by L-methionine would thereby reduce cellular damage induced by ischemia–reperfusion and decrease eventually the production of other molecules acting as injury signals, which in turn may lead to leukocyte activation. In this regard, platelet activation factor and leukotrienes that increased locally during ischemia–reperfusion cycles induce granulocyte activation and release of oxygen species (36–38). Also, ischemia increases the levels of xanthine and xanthine oxidase, which in the presence of oxygen result in the production of highly reactive prooxidants (39,40).

Another protective mechanism that deserves further investigation involves an indirect effect of L-methionine, via the intracellular formation of glutathione, a well known protector against ischemic damage (41,42), and/or the synthesis of *S*-adenosylmethionine, which has been shown to maintain cell membrane integrity in different experimental models of cell injury (43,44). Both actions might contribute indirectly to reduce the extent of cellular lesion and to attenuate the release of leukocyte activating mediators.

Our data confirm that leukocyte peroxidative activity increases shortly after coronary ligation and reperfusion, and that L-methionine exerts a noticeable inhibitory effect over peroxidative activity both in local and peripheral granulocytes. This observation may contribute to the explanation of the protective effect of this amino acid in some experimental models of myocardial ischemia. The results of our study might also have some therapeutic implications for the prevention and treatment of ischemic lesions in human patients, although care must be taken when extrapolating from experimental data obtained in animal models. In this regard, it is worth noting that the use of flow cytometric techniques based upon coronary blood sampling makes it feasible to design protocols for the assessment of lesion risk and the monitoring of therapy in conditions of myocardial ischemia and reperfusion.

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