Thromboxane B₂ in Cardiac Lymph
Effect of Superoxide Dismutase and Catalase During Myocardial Ischemia and Reperfusion

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Reactive oxygen species such as superoxide anion have been implicated as important agents involved in causing cell death in the setting of myocardial ischemia and reperfusion.¹,² Leukocytes may exert their membrane-damaging effects by the production of these reactive oxygen species as well as by the alteration of microvascular and endothelial function. Studies have demonstrated favorable effects of free radical scavengers on recovery of myocardial function and infarct reduction after reversible regional ischemia.¹,³,⁴ In addition, peripheral blood polymorphonuclear leukocytes synthesize lipoxygenase and cyclooxygenase derivatives on exposure to chemotactic stimuli, and these autacoids are known to induce vasoconstriction, platelet aggregation, and direct tissue toxicity.⁵,⁶

Thus, the production of reactive oxygen species and the production of oxygenase-derived autacoids represent two potential independent mechanisms by which tissue injury can be induced by leukocyte activation. These functions, however, may not be totally independent. The work of Hemler and Lands⁷ has suggested that fatty acid oxygenases require a hydroperoxide activator. Therefore, augmentation of the production of molecular oxygen species may potentially amplify oxygenase enzyme activity. Work by Shappell et al⁸ demonstrated that both superoxide dismutase (SOD) and catalase (CAT) inhibit arachidonate-induced platelet function, suggesting the converse (i.e., that scavenging of reactive oxygen species may inhibit arachidonate-dependent function).

Our past studies of conscious animals demonstrated a striking increase in cardiac lymph immunoreactive thromboxane B₂ (TXB₂) concentration after ischemia and reperfusion following a coronary artery occlusion.⁹ In view of the potential relation between molecular oxygen species and cyclooxygenase-derived autacoid production,⁷ we chose to examine the effects of SOD and CAT on the rise in TXB₂ formation observed with myocardial ischemia and reperfusion. The results provide in vivo evidence that SOD and CAT in combination prevent the rise in TXB₂ appearance in cardiac lymph observed during ischemia and reperfusion. SOD alone is also effective, but CAT alone is ineffective in preventing the TXB₂ generation.

Materials and Methods

The instrumentation of conscious dogs and cannulating cardiac lymph ducts have been described

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This study was based on 31 healthy mongrel dogs of either sex weighing 16–24 kg. At least 2 days after surgery, cardiac lymph samples were collected every 30 minutes on ice in polypropylene tubes with 0.1 μM prostaglandin E₁, 10 μM indomethacin, 0.5 mM theophylline, and 5 mM EDTA and then centrifuged at 8,000g for 15 minutes at 4° C. The supernatant from each tube was frozen in dry ice–acetone and stored at −70° C. Radioimmunoassay was performed with [125I]TXB₂ and rabbit anti–TXB₂ (Advanced Magnetics, Cambridge, Massachusetts).

TXB₂ was measured in cardiac lymph during the following protocols: 1) 5-minute occlusion followed by reperfusion in the presence of 0.9% saline infusion, 2) 60-minute occlusion followed by reperfusion in the presence of 0.9% saline infusion, 3) 60-minute occlusion followed by reperfusion in the presence of SOD infusion, 4) 60-minute occlusion followed by reperfusion in the presence of CAT infusion, and 5) 60-minute occlusion followed by reperfusion in the presence of SOD and CAT infusion.

Animals receiving drug were infused with either 0.06 mg/kg/min CU-ZN-SOD purified from bovine liver (activity, 3,300 units/mg protein; DDI Pharmaceuticals, Mountain View, California) plus 0.06 mg/kg/min CAT purified from bovine liver (activity, 11,000 units/mg protein; Sigma Chemical, St. Louis, Missouri) in saline, SOD alone, CAT alone, or an equal volume of saline alone. The infusion was administered through a left atrial catheter at 0.2 ml/min starting 15 minutes before coronary artery occlusion and continuing until 15 minutes after the onset of reperfusion or 75 minutes after the onset of reperfusion (for study of effects of SOD for a longer term). The occlusion period was 60 minutes, and the reperfusion was continuous thereafter. Restoration of blood flow was confirmed by noting the immediate reactive hyperemic response detected by the coronary flow velocity probe.

**Data Analysis**

The data were analyzed by BMDP5V, an extended repeated measures program. Further analysis by either paired *t* test or *F* test determined significant differences between the various parameters with significance accepted at *p*<0.05. Values are expressed as mean±SEM.

**Results**

The multivariate analysis of repeated measures for incomplete data was chosen by our biostatistician as the appropriate method since our data did not have compound symmetry and, hence, were not acceptable for univariate analysis. The Wald tests of significance of fixed effects and covariates were used, and a value of *p*<0.02 indicated statistical significance for treatment-time interactions. Further analyses by paired *t* test compared the effect of treatment at each time as well as between time periods.

As previously described, coronary occlusions lasting 60 minutes followed by reperfusion result in elevation of TXB₂ excretion into cardiac lymph (Figures 1 and 2). Elevated TXB₂ excretion was not obvious until the onset of reperfusion (data not shown) but rose to significant levels (*p*<0.01) immediately at the onset of reperfusion. Peak TXB₂ excretion rate was reached, and significant increases were maintained within the first 2 hours of reperfusion. The increase in TXB₂ release rate was paralleled by both an increase in cardiac lymph flow (*p*<0.01) and TXB₂ concentration (*p*<0.05) in cardiac lymph, both of which persisted throughout the 2-hour period of reperfusion (Figure 1). Coronary blood flow studies demonstrated identical reactive hyperemia responses in all groups; coronary flow returned to normal in 10–15 minutes (data not shown).
Considerable data exists within the literature suggesting that endothelial injury in the constricted coronary artery can occur consequent to coronary artery instrumentation and constriction; therefore, we performed an experiment in which coronary occlusion was maintained for only 5 minutes but in which the remainder of the protocol was identical with that used for longer occlusions. No elevation in TXB₂ concentration occurred consequent to a 5-minute coronary occlusion in any of the animals tested (Figures 2 and 3). Thus, it seems unlikely that coronary instrumentation per se is resulting in endothelial damage sufficient to activate blood elements to excrete cyclooxygenase products.

Infusion of SOD-CAT prevented a significant rise in TXB₂ excretion rate observed in the untreated animal (p>0.1; Figures 1–3). SOD-CAT treatment also prevented the increase in cardiac lymph flow and TXB₂ concentration in cardiac lymph (Figure 1). Independent effects of SOD and CAT infusion illustrated that SOD alone reduced TXB₂ generation so that no significant rise in TXB₂ formation could be observed when compared with baseline (p>0.1) (Figure 3). At 90 minutes of reperfusion, the values were not significantly different from those observed with SOD and CAT (p>0.1) combined and were significantly lower (p<0.05) than those in the untreated control.

SOD infusions (in the presence or absence of CAT) are uniformly effective in reducing TXB₂ formation during the first 90 minutes after reperfusion. After 90 minutes, there was considerable variability. In a number of animals, partial or complete return of TXB₂ concentration to levels observed in untreated animals with ischemia and reperfusion was observed (Figures 1–3), and cardiac lymph flow and TXB₂ concentrations also increased (Figure 1). Because the original protocol called for administration of the potential therapeutic agents to cease 15 minutes after the onset of reperfusion, it was reasoned that potentially effective agents may have been cleared from the circulation within 2 hours. Therefore, we examined the effects of an SOD infusion that began 15 minutes before the onset of ischemia and persisted for 75 minutes after the onset of reperfusion. Cessation of this prolonged SOD infusion resulted in a suppression of TXB₂ for as long as 3 hours, after which there was a trend toward increased excretion (Figure 4).
Molecular oxygen species are potentially a major factor in the genesis of myocardial ischemia-reperfusion injury. Several pathways may be responsible for the generation of oxygen free radicals at the time of reperfusion including 1) production during lipoygenase or cyclooxygenase activation, 2) release of superoxide anions by activated neutrophils migrating into ischemically damaged tissue, 3) accumulation of hypoxanthine and xanthine during myocardial ischemia catalyzed to uric acid by xanthine oxidase, and 4) oxidation of tissue catecholamines. In our experiments, an administration of SOD and CAT 15 minutes before a 60-minute occlusion that continued for 15 minutes into reperfusion resulted in decreasing concentration of cardiac lymph TXB2 concentration. Since SOD and CAT act extracellularly, it is likely that the molecular oxygen species affected were in the extracellular compartment and, therefore, were, at least in part, of leukocyte origin.

We suggest that TXB2 formation begins during ischemia and relates, in part, to the length of ischemia since coronary occlusion alone (in short duration) does not seem to result in increases in TXB2 formation. The initial reperfusion collection period frequently demonstrated equal or higher TXB2 excretion rates than future reperfusion periods. This suggests that, at least in part, the initial TXB2 has been generated during the ischemic period and appears in cardiac lymph at the onset of reperfusion as previously suggested by our laboratory on examination of creatine phosphokinase appearance in cardiac lymph. The fact that the TXB2 excretion remains elevated and, in some cases, continues to rise suggests that an ongoing process during reperfusion continues to promote TXB2 excretion.

This study does not directly speak to the origin of the thromboxane production; this autacoid is produced by both neutrophils and platelets, and each of these blood elements is thought to be pertinent to the pathophysiology of myocardial ischemia. However, despite the fact that coronary blood flow returns to normal within 10–15 minutes, increased TXB2 generation is associated with increased coronary lymph flow during the 2 hours of reperfusion. This finding suggests the presence of a pathophysiological factor that alters microvascular permeability associated with the 1-hour ischemia-reperfusion protocol. In view of the postulated pathophysiological role of neutrophils in alterations of the microvascular endothelium in ischemic damage, the possibility that a neutrophilic origin of some of the observed TXB2 appears likely. It would be tempting to speculate that increased neutrophil activation results in microvascular damage and also results in increased TXB2 production. These results are compatible with the suggestions of others that oxygen free radical generation is an important part of neutrophil-induced dysfunction and damage in tissue and in the generation of lipid-derived autacoids.

In this study, SOD is the active agent in reducing TXB2 formation in this ischemia-reperfusion proto-

**Discussion**

A large series of studies has been done to demonstrate that free radical scavengers exert favorable effects on a variety of acute ischemic models including myocardial infarction and stunned myocardium. The original work in Lucchesi's laboratory (see Reference 1 for summary) suggested that SOD plus CAT was effective in preventing the additional injury occurring during reperfusion after a coronary occlusion. Subsequently, it has become clear that SOD is the major operant in the reduction of infarct size and that its protective effect only requires its presence during the time of reperfusion. It is important to emphasize that the effect of SOD plus CAT or SOD alone is highly dependent on the protocol used in the studies. Measurement of infarct size after longer periods of occlusion or after longer periods of reperfusion shows variable effects with regard to the usefulness of free radical scavengers. It is likely that free radical scavengers are required for some significant period of time during reperfusion to ultimately reduce the size of a myocardial infarction and that shorter periods of treatment may only delay but not decrease infarct size. At any rate, the concentration used in this study was identical with that which minimized infarct size and markedly improved the recovery of myocardial function after a 15-minute reversible coronary occlusion (i.e., reduced stunned myocardium).

In previous studies from our laboratory, we observed marked elevations in TXB2 concentration seen in cardiac lymph subsequent to a coronary occlusion and demonstrated that this could be blocked by either ibuprofen or prostacyclin. Since thromboxane formation is both accompanied by and may be potentiated by the generation of oxygen free radicals, this study was designed to examine the effects of a free radical scavenger with known salutary effects on myocardial ischemia on the generation of thromboxane.

**Figure 4.** Thromboxane B2 (TXB2) concentrations in cardiac lymph at times shown during reperfusion after a 60-minute coronary occlusion with extended superoxide dismutase treatment for the first 75 minutes of reperfusion. N, number of animals. Error bars represent SEM.
col. It is important to point out that the absence of an effect of CAT does not necessarily belie its function as an oxidative scavenger; it is possible that the endogenous CATs and peroxidases present within the cells in question and within the circulation are sufficient to exert an optimal effect.

In our experiments, TXB₂ and cardiac lymph flow begin to rise within 3 hours after discontinuing the SOD infusion; this occurrence further suggests that ongoing SOD treatment may be necessary for protection for a greater length of time. The infusion time required for ultimate protection from a 1-hour ischemic injury is not determined by this study. Recent work by Simpson et al. has suggested that periods up to 72 hours are required for ultimate prevention of neutrophil-derived damage.

Therefore, in addition to the direct toxic effect of peroxidative tissue injury, oxidative stress may also catalytically stimulate the production of vasoactive autacoids that may induce vasoconstriction and platelet aggregation. Together with the microvascular effects of leukocyte adherence and aggregation, these effects may induce severe endothelial damage and microvascular dysfunction associated with occlusion-reperfusion injury.

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References


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