Active Oxygen Species Play a Role in Mediating Platelet Aggregation and Cyclic Flow Variations in Severely Stenosed and Endothelium-Injured Coronary Arteries


A canine model with cyclic flow variations (CFVs) in stenosed and endothelium-injured coronary arteries was used to examine the role of active oxygen species in platelet aggregation in vivo. We studied 90 anesthetized dogs in which the pericardial cavity was opened and the heart was exposed. The velocity of blood flow in the left anterior descending coronary artery (LAD) was monitored by a pulsed Doppler flow probe. In 67 dogs, the LADs were stenosed by applying external constrictors at the site where the endothelium was mechanically injured. CFVs developed in all 67 dogs. Treatment with the antioxidants recombinant human copper-zinc superoxide dismutase (r-h-CuZnSOD), recombinant human manganese superoxide dismutase (r-h-MnSOD), and catalase eliminated platelet aggregation-associated coronary CFVs in 63%, 62%, and 64% of animals, respectively. Intravenous infusion of epinephrine restored CFVs in most dogs. Ketanserin, a serotonin (5-hydroxytryptamine,) receptor antagonist, abolished epinephrine-restored CFVs and eliminated CFVs in dogs in which CFVs had not been eliminated by free radical scavengers. In an additional 23 dogs, the LADs were stenosed but not mechanically injured. For control studies, saline was infused into the LADs of 5 dogs. Xanthine/xanthine oxidase was infused into the LADs of 8 dogs and induced CFVs in 4. Hydrogen peroxide was infused into the other 10 dogs and induced CFVs in 9. Histological analysis of the coronary artery revealed that the intima was significantly injured by the infusion. In ex vivo platelet aggregation studies, the in vivo treatment with r-h-CuZnSOD, r-h-MnSOD, and catalase significantly inhibited platelet aggregation induced by platelet-activating factor. Thus, active oxygen species are involved in mediating platelet aggregation and cyclic flow variations in stenosed and endothelium-injured canine coronary arteries in vivo. (Circ Res. 1993;73:952-967.)

KEY WORDS • recombinant human copper-zinc superoxide dismutase • recombinant human manganese superoxide dismutase • epinephrine • ketanserin • active oxygen species • platelet aggregation

Acute vascular injury caused by balloon angioplasty usually involves platelet deposition, thrombosis, and vasospasm.1-3 These changes may lead to acute occlusion of coronary arteries and may contribute to chronic restenosis after successful angioplasty.4-6 Recent studies have suggested that some platelet-derived factors may be involved in acute platelet deposition and chronic restenosis of injured coronary arteries.7-10 However, the precise mechanisms are not clear.

Active oxygen species originate from the univalent reduction of molecular oxygen in cellular metabolism.11

Under normal conditions, the accumulation of active oxygen species is limited by antioxidant enzymes and other nonenzymatic compounds. In the injured vessel, leukocyte infiltration into the vessel wall and platelet accumulation on the injured vascular surface could result in the production of excessive amounts of active oxygen species,12-14 which are highly reactive and potentially toxic. The arterial endothelium may also be a source of active oxygen species. Published data suggest that active oxygen species may alter endothelial function, influence vessel tone, and cause reperfusion injury of the myocardium.15-17 In addition, active oxygen species may alter platelet aggregation in vitro.18-25

In the present study, we used an experimental canine model to test the hypothesis that active oxygen species are involved in mediating platelet aggregation in severely stenosed and endothelium-injured coronary arteries in vivo. Three antioxidant enzymes, recombinant human copper-zinc superoxide dismutase (r-h-CuZnSOD), recombinant human manganese superoxide dismutase (r-h-MnSOD),26,27 and catalase, were used to
Fig 1. Experimental protocol is shown. CFV indicates cyclic flow variation; EPI, epinephrine; KET, ketanserin; SOD, superoxide dismutase; and X/XO, xanthine/xanthine oxidase.

test the hypothesis in dogs with severely stenosed and endothelium-injured coronary arteries.

Materials and Methods
All procedures used in this study were conducted according to the principles of the American Physiological Society and were approved by the Institutional Animal Care and Use Committee at the Texas Heart Institute, Houston.

Surgical Preparation
Mongrel dogs (n=90) weighing 25 to 35 kg were anesthetized with sodium pentobarbital (30 mg/kg IV), intubated, and placed on mechanical respirators (model 60, Harvard Apparatus, South Natick, Mass). Plastic catheters were placed in a carotid artery for monitoring aortic pressure and in a jugular vein for administering drugs and fluids. A thoracotomy was performed in the
### Hemodynamics in Dogs With Cyclic Flow Variations Before and After Treatments

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<th>Heart Rate (bpm)</th>
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bpm indicates beats per minute; FV, flow velocity in the coronary artery; CFVs, cyclic flow variations; r-h-CuZnSOD, recombinant human copper-zinc superoxide dismutase (SOD); EPI, epinephrine; r-h-MnSOD, recombinant human manganese SOD; X/XO, xanthine/xanthine oxidase; and H₂O₂, hydrogen peroxide.

*Reported as a percentage of control.
†P<.05 compared with within-group control value.
Table (Continued)

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Experimental Procedures

Baseline hemodynamics, including heart rate, systolic and diastolic aortic pressures, and phasic and mean blood flow velocities in the LAD, were recorded on an eight-channel recorder (model 3000, Gould, Inc, Cleveland, Ohio). Then, in 67 dogs, the endothelium of the exposed LAD was injured by gently squeezing the artery 10 to 20 times with cushioned forceps. A plastic constrictor was placed around the injured artery to reduce the phasic arterial blood flow velocity to 50% to 60% of the baseline level. Subsequently, cyclic flow reductions developed as a result of recurrent platelet aggregation and dislodgement on the injured endothelial sur-

Fig 2. Bar graph shows frequency of abolition of cyclic flow variations (CFVs) by different treatments. *P<.05 compared with recombinant human copper-zinc superoxide dismutase.

Fig 3. Bar graphs show the frequency of restoration of cyclic flow variations (CFVs) by epinephrine (EPI) (A) and the dosage of EPI required to restore CFVs (B) after elimination of CFVs by different treatments. SOD indicates superoxide dismutase; CATAL, catalase; and KETAN, ketanserin. *P<.05 compared with single treatment.
face. Further studies were performed according to the protocol shown in Fig 1.

**Group 1**. In 15 dogs, r-h-CuZnSOD (3600 U/mg) was intravenously administered 30 minutes after cyclic flow variations were established. Dogs in group 1A (n=7) received 2 mg/kg r-h-CuZnSOD in bolus and 0.8 mg/kg (kg.h) in continuous infusion. Dogs in group 1B (n=8) received 6 mg/kg r-h-CuZnSOD in bolus and 6 mg/kg (kg.h) in continuous infusion. r-h-CuZnSOD was administered at two different doses to observe differing responses. The dogs in both subgroups were monitored for 2.5 hours to observe changes in frequency and severity (detected by the nadir flow) of cyclic flow variations.

Further studies were then performed on group 1B. If cyclic flow variations were abolished in these dogs, epinephrine was intravenously infused in increments of 2.4, 4.5, 9, and 17 μg/min to restore cyclic flow variations. Epinephrine was infused to determine whether r-h-CuZnSOD could protect against epinephrine-restored cyclic flow variations. Epinephrine augments platelet aggregation; it accumulates in the myocardium and systemic circulation in patients with acute coronary artery disease syndromes, and in this experimental animal model, it restores cyclic flow variations after they have been abolished by aspirin or a single antagonist for thromboxane A$_2$ and serotonin.31-33 Dogs in which cyclic flow variations were restored by epinephrine then received ketanserin, a serotonin receptor antagonist and effective inhibitor of cyclic flow variations (Janssen Pharmaceutical Co, Beerse, Belgium). If cyclic flow variations were again abolished, epinephrine was again administered to examine the degree to which r-h-CuZnSOD and ketanserin could protect against the restoration of cyclic flow variations by epinephrine. Restored cyclic flow variations were monitored for 30 minutes to ensure their persistence. We recorded the dose of epinephrine required to restore cyclic flow variations. If cyclic flow variations were not restored, the highest administered dose of epinephrine was recorded.

Dogs in group 1B in which cyclic flow variations were not abolished received an intravenous bolus of ketanserin (0.25 mg/kg). (Combining drugs with different actions may enhance the effect of each drug; this practice is often used to treat patients.) Thirty minutes after cyclic flow variations were abolished in the ketanserin-treated dogs, epinephrine was administered to try to restore the variations.

**Group 2**. In 13 dogs, r-h-MnSOD (3600 U/mg) was intravenously administered at 2 mg/kg in bolus 30 minutes after coronary cyclic flow variations were established. The dose of r-h-MnSOD was based on previous studies.20 r-h-MnSOD is relatively positively charged at a physiological pH level and possibly has a higher cellular binding affinity than r-h-CuZnSOD, which is relatively negatively charged at a physiological pH level (personal communication, Dr Amnon Gonenne, Bio-Technology, New York, NY). Dogs were monitored for 2.5 hours to observe changes in frequency and severity of cyclic flow variations. If cyclic flow variations were abolished, epinephrine was administered. Thirty minutes after cyclic flow variations were restored by epinephrine, ketanserin was administered. If ketanserin abolished cyclic flow
Fig 5. (This page and following page). A, Photomicrograph of left anterior descending coronary artery (LAD) after xanthine/xanthine oxidase infusion shows fragmentation of internal elastic lamina (arrow) with accumulation of platelets (p) and erythrocytes (e) in the media (M) (hematoxylin-eosin, 1-μm thick, glycol methacrylate sections, original magnification ×50). B, Scanning photomicrograph of LAD shows endothelial disruption (arrowhead) and platelet aggregation (p) (bar=50 μm, original magnification of boxed area ×4). C, Photomicrograph of left circumflex coronary artery (LCCA) (control) shows intact media (M), internal elastic lamina (arrow), and endothelium (hematoxylin-eosin, 1-μm thick, glycol methacrylate sections, original magnification ×50). D, Scanning photomicrograph of LCCA (control) shows intact endothelium (bar=50 μm; original magnification of boxed area, ×4).

variations, epinephrine was administered 30 minutes later in increasing doses, as described above. In dogs in which cyclic flow variations were not abolished by r-h-MnSOD, ketanserin was intravenously administered at 0.25 mg/kg. Epinephrine was also administered 30 minutes after the elimination of cyclic flow variations. These interventions were conducted to determine the effectiveness of r-h-MnSOD alone and in combination with ketanserin to abolish cyclic flow variations.

Group 3. In 11 dogs, 30 minutes after cyclic flow variations were established, catalase (bovine, 18600 U/mg) was administered as an intravenous bolus (2 mg/kg) and as a continuous infusion at 2 mg/(kg·h). Catalase converts hydrogen peroxide to water and oxygen. These treatments were to evaluate the effect of catalase on cyclic flow variations. Dogs were monitored for 2.5 hours to observe changes in cyclic flow variations. If the cyclic flow variations were abolished, epinephrine was infused in the manner described above. After epinephrine restored cyclic flow variations, ketanserin was administered. Then, epinephrine was administered in increasing concentrations. In dogs in which cyclic flow variations were not abolished by treatment with catalase, ketanserin was given. If the addition of ketanserin abolished cyclic flow variations in these dogs, epinephrine was then administered.
Group 4. In 7 control dogs, ketanserin was administered 3 hours after cyclic flow variations were established, as a control for the above three groups. Thirty minutes after cyclic flow variations were abolished, epinephrine was infused at increasing concentrations to try to restore cyclic flow variations. Restored cyclic flow variations were monitored for 30 minutes to ensure their persistence.

Group 5. The direct effect of active oxygen species on coronary flow was studied in 23 dogs. In 18 dogs (group 5A), an external constriction was placed around the coronary artery, but forceps were not used to squeeze and injure the artery. A plastic catheter was inserted into the artery through a branch proximal to the constrictor, and saline was infused into the artery for 30 minutes. In 8 of these dogs, a mixture of xanthine/xanthine oxidase (a generator of superoxide anion) was infused through the coronary catheter in increasing concentrations (4 μg xanthine and 4 mU xanthine oxidase per minute, 8 μg xanthine and 8 mU xanthine oxidase per minute, 12 μg xanthine and 12 mU xanthine oxidase per minute, and 16 μg xanthine and 16 mU xanthine oxidase per minute). The estimated superoxide generation was 2 to 10 nmol/L. In the other 10 dogs in this subgroup, hydrogen peroxide was infused into the coronary catheter at a concentration of 1.5 mg/min (estimated blood concentration, 0.44 mmol/L). Dosages of xanthine/xanthine oxidase and hydrogen peroxide were chosen on the basis of previous experiments. The velocity of coronary blood flow was continuously monitored during the infusion. If cyclic flow variations developed, the flow velocity was monitored for an additional 30 minutes to ensure their consistency. In 6 dogs treated with hydrogen peroxide, catalase was administered as an intravenous bolus of 2 mg/kg and then as a continuous infusion of 2 mg/(kg·h) to try to abolish the hydrogen peroxide–induced cyclic flow variations. If the cyclic flow variations were abolished, the infusion of
catalase was discontinued 30 minutes later to try to restore them. In dogs in which cyclic flow variations were restored, ketanserin was given as an intravenous bolus of 0.25 mg/kg to try to abolish restored cyclic flow variations.

In 2 dogs treated with xanthine/xanthine oxidase and in 2 dogs treated with hydrogen peroxide, the infused coronary artery and the normal circumflex coronary artery were perfused with 3% glutaraldehyde for 10 minutes.

Arteries were then divided into two groups. Arteries to be examined under the light microscope were post-fixed in 10% buffered formalin. Arteries for scanning electron microscopy were dehydrated through graded acetones and then critical point-dried. Dried tissues were coated with gold-palladium (Denton Vacuum, DV-502A) and examined with a Zeiss DSM 960 microscope. For light microscopy, tissue was dehydrated through graded acetones and embedded in glycol methacrylate. Sections of 1-μm thickness were obtained with an LKB 2218 Historange microtome and were stained with hematoxylin and eosin.

In 5 dogs (group SC), saline was infused into the LAD at 0.2 mL/min for 3 hours after placement of the constrictor. After infusion of saline, the constricted segment of LAD was collected in the same manner as described above for light microscopic examination.

Group 6. To explore the relative contribution of superoxide anions and hydrogen peroxide to the development of cyclic flow variations, 21 additional dogs were studied. The dogs were prepared, and cyclic flow variations were established in the same manner as for dogs in groups 1 through 3. r-h-MnSOD (n=10) or catalase (n=11) was initially administered. If the treatment failed to eliminate cyclic flow variations, either catalase or r-h-MnSOD was added. The dosage and route of administration of r-h-MnSOD or catalase was the same as described for groups 2 and 3.

Measurements of r-h-CuZnSOD and r-h-MnSOD in the Circulation

The concentrations of r-h-CuZnSOD and r-h-MnSOD in the circulation of dogs in groups 1A and 2 were measured before and after treatment with each reagent. Blood was collected from the carotid arterial catheter and placed into serum separation tubes (Becton Dickinson Vacutainer System, Rutherford, NJ). Serum was separated and stored at -20°C until analyzed. Quantitation of the enzymes was performed by a radioimmunoassay with monoclonal antibodies to r-h-CuZnSOD and r-h-MnSOD.

Ex Vivo Platelet Aggregation Studies

Ex vivo platelet aggregation was studied before and 10 minutes after the administration of r-h-CuZnSOD, r-h-MnSOD, catalase, and ketanserin to dogs in groups 1B, 2, 3, and 4. Blood samples were collected in plastic tubes containing a 3.8% solution of sodium citrate (9 vol blood:1 vol sodium citrate). Platelet-rich plasma was obtained by centrifuging blood samples at 200g for 20 minutes, and platelet-poor plasma was obtained by centrifuging the residual blood at 3000g for 10 minutes. The platelet count in platelet-rich plasma was adjusted to 300 000/mm³. A four-channel platelet aggregometer (model PAP-4, Bio-Data, Horsham, Pa) was used for the assay. Agonists and their final concentrations were ADP at 5 to 20 μmol/L, U46619 (a thromboxane mimetic) at 40 to 160 ng/mL, serotonin at 0.5 to 2 μmol/L, and platelet-activating factor at 0.01 to 1.0 μmol/L. Because canine platelets do not aggregate in response to U46619 or serotonin alone, epinephrine was added at 10 μmol/L before the addition of U46619 or serotonin. The degree of platelet aggregation was reported as a percentage of maximal increase of light transmission in platelet-rich plasma over that in platelet-poor plasma.

Materials

The antioxidant enzymes r-h-CuZnSOD and r-h-MnSOD were kindly provided by Bio-Technology General Corp, New York, NY. The serotonin receptor antagonist ketanserin was generously provided by Janssen Pharmaceutical Co, Beerse, Belgium. Catalase, ADP, serotonin, xanthine/xanthine oxidase, and platelet-activating factor were purchased from Sigma Chemical Co, St Louis, Mo. U46619 was purchased from Calbiochem Corp, La Jolla, Calif. Epinephrine was purchased from Elkins-Sinn, Inc, Cherry Hill, NJ. Hydrogen peroxide was purchased from Humco Lab, Texarkana, Tex.

Statistical Analyses

All values are expressed as mean±SEM. The χ² test and Fisher’s exact test were used to compare the frequency of abolition and restoration of cyclic flow variations after different treatments. A one-way analysis of variance with repeated measurements was used to compare the hemodynamic values obtained at different time periods. Student’s t test was used to compare doses of epinephrine required to restore cyclic flow variations, the frequency of cyclic flow variations before and after...
Fig 7. A, Photomicrograph of left anterior descending coronary artery (LAD) after hydrogen peroxide infusion shows fragmentation of internal elastic lamina (arrow) with smooth muscle cell vaculization (v) in the media (M) (hematoxylin-eosin, 1-μm thick, glycol methacrylate sections, original magnification ×50). B, Scanning photomicrograph of LAD shows loss of endothelial continuity and surface covered by fibrin (arrowhead), enmeshed erythrocytes (e), and platelet aggregation (p) (bar=50 μm, original magnification of boxed area ×4). C, Photomicrograph of left circumflex coronary artery (LCCA) (control) shows intact media (M), internal elastic lamina (arrow), and endothelium (hematoxylin-eosin, 1-μm thick, glycol methacrylate sections, original magnification ×50). D, Scanning photomicrograph of LCCA (control) shows intact endothelium (bar=50 μm, original magnification of boxed area ×4).

Results

Cyclic flow variations developed in all 67 dogs after endothelial injury and external constriction of the LAD. The reduction of coronary flow velocity caused by treatment, the concentration of circulating r-h-MnSOD, and the degree of platelet aggregation in different groups of animals. A value of \( P<.05 \) was considered significant.
external constriction was similar among different groups of animals (phasic coronary flow velocity reduced to 59±6% of baseline level in group 1A, to 56±3% in group 1B, to 52±4% in group 2, to 50±3% in group 3, and to 56±6% in group 4; P>.05; Table). The frequency and severity of initial cyclic flow variations (as determined by nadir flow) were also similar among the five groups of animals (Table). Heart rates and aortic pressures did not change significantly after the development of cyclic flow variations (Table).

**Effect of r-h-CuZnSOD, r-h-MnSOD, Catalase, and Ketanserin on Spontaneous Cyclic Flow Variations**

The administration of r-h-CuZnSOD at 2 mg/kg bolus and 0.8 mg/kg continuous infusion eliminated cyclic flow variations in 3 of the 7 dogs in group 1A. Increasing the dose of r-h-CuZnSOD to a 6 mg/kg bolus and continuous infusion at 6 mg/(kg·h) eliminated cyclic flow variations in 5 of 8 dogs in group 1B. Cyclic flow variations were abolished in 8 of 13 dogs in group 2 by a 2 mg/kg bolus dose of r-h-MnSOD. This abolition rate was similar to that achieved by treatment with a high dose of r-h-CuZnSOD but slightly higher than that achieved by low-dose r-h-CuZnSOD treatment (Fig 2).

In group 3, catalase abolished cyclic flow variations in 7 of 11 dogs. These results are similar to those obtained with r-h-MnSOD. Ketanserin completely eliminated cyclic flow variations in those dogs in which cyclic flow variations were not abolished by initial treatment with r-h-CuZnSOD, r-h-MnSOD, or catalase. In group 4 dogs, ketanserin abolished cyclic flow variations in all 7 dogs (Fig 2). Heart rates and aortic pressures were not significantly affected by treatment with r-h-CuZnSOD, r-h-MnSOD, catalase, or ketanserin (Table).

**Effect of Single Treatment with r-h-CuZnSOD, r-h-MnSOD, Catalase, or Ketanserin on Epinephrine-Restored Cyclic Flow Variations**

Epinephrine was infused in dogs in which cyclic flow variations had been abolished by treatment with active oxygen species scavengers or ketanserin. Epinephrine restored cyclic flow variations in 5 of 5 dogs in group 1B after their elimination by r-h-CuZnSOD, in 7 of 7 dogs in group 2 after their elimination by r-h-MnSOD, in 5 of 6
dogs in group 3 after their elimination by catalase, and in 6 of 7 dogs in group 4 after their elimination by ketanserin (Fig 3A). The frequency of epinephrine-restored cyclic flow variations was similar to that established initially (Table). The severity of the restored cyclic flow variations (determined by nadir flow) was also the same as that observed in initial flow variations (Table). The dose of epinephrine required to restore cyclic flow variations was slightly but not significantly higher in catalase-treated dogs than in other dogs (Fig 3B).

**Effect of the Combination of r-h-CuZnSOD, r-h-MnSOD, or Catalase With Ketanserin on Epinephrine-Restored Cyclic Flow Variations**

Ketanserin abolished epinephrine-restored cyclic flow variations. Ketanserin also abolished cyclic flow variations in all dogs in which cyclic flow variations were not eliminated by treatment with r-h-CuZnSOD, r-h-MnSOD, or catalase. After elimination of cyclic flow variations by combined treatment (r-h-CuZnSOD, r-h-MnSOD, or catalase and ketanserin), epinephrine restored cyclic flow variations in 3 of 7 dogs in group 1B, 4 of 8 dogs in group 2, and 0 of 7 dogs in group 3. The frequency of the restored cyclic flow variations was similar to the frequency of initial cyclic flow variations and also to the frequency of cyclic flow variations that had been restored after elimination by single treatment (Table). However, the severity of the restored cyclic flow variations was much less than the severity of initial ones (Table). The dose of epinephrine required to restore cyclic flow variations that had been eliminated by combination treatment was significantly higher than
that required to restore cyclic flow variations that had been abolished by single treatment (Fig 3B).

**Effects of Xanthine/Xanthine Oxidase and Hydrogen Peroxide on Coronary Flow and Coronary Arterial Wall**

In group 5 dogs, coronary arteries were stenosed by an external constrictor (Table); however, the endothelium was not injured with forceps. Infusion of saline into the coronary artery for 30 minutes did not significantly change coronary flow (Table). Infusion of 4 μg xanthine and 4 mU xanthine oxidase per minute into the coronary arteries induced cyclic flow variations in 4 of 8 dogs. The xanthine/xanthine oxidase–induced cyclic flow variations were not persistent and faded immediately (Fig 4A). Light and scanning electron microscopic examination of the coronary arteries revealed patches of endothelial cell loss or necrosis in the xanthine/xanthine oxidase–infused segment (Fig 5A and 5B) when compared with the normal left circumflex coronary artery in the same dog (Fig 5C and 5D). Cyclic flow variations never developed in the other 4 dogs, and ventricular fibrillation occurred after high doses of xanthine/xanthine oxidase were given.

Infusion of hydrogen peroxide into the coronary arteries induced persistent cyclic flow variations in 9 of 10 dogs (Fig 4B). Hydrogen peroxide induced more frequent cyclic flow variations than did xanthine/xanthine oxidase (Fig 6). Catalase was administered to 6 dogs with hydrogen peroxide–induced cyclic flow variations. Treatment with catalase abolished cyclic flow variations in 4 dogs (67%). Ketanserin eliminated the hydrogen peroxide–induced cyclic flow variations in the other 2 dogs. In the 4 dogs in whom catalase eliminated cyclic flow variations, discontinuation of catalase infusion restored cyclic flow variations with the same frequency and severity in approximately 30 minutes in all dogs (Fig 4C). Ketanserin was then given to these animals, and it abolished the cyclic flow variations. Examination of the coronary artery with light and scanning electron microscopy demonstrated a diminished endothelial circumference and marked fragmentation and separation of the media where hydrogen peroxide was infused (Fig 7A and 7B). The left circumflex coronary artery from the same dog appeared normal (Fig 7C and 7D). The 5 additional dogs (group 5C) into whose LADs saline was infused for 3 hours after placement of the constrictor did not develop cyclic flow variations. The constricted segment was examined with light microscopy; it revealed minor endothelial injury with intact internal elastic lamina in some areas and intact endothelium in other areas (Fig 8A and 8B).

**Relative Effects of r-h-MnSOD and Catalase on Cyclic Flow Variations**

In group 6, initial treatment with r-h-MnSOD failed to eliminate cyclic flow variations in 5 of 10 dogs. The addition of catalase, however, completely abolished the remaining cyclic flow variations in those dogs (Fig 9). Initial treatment with catalase did not eliminate cyclic flow variations in 5 of 11 dogs. Nor did the addition of r-h-MnSOD abolish the cyclic flow variations (Fig 9). The frequency of cyclic flow variations after the additional treatment with r-h-MnSOD was somewhat greater than the frequency after the initial treatment with catalase, although the difference was not statistically significant.

**Circulating Concentrations of r-h-CuZnSOD and r-h-MnSOD**

The concentrations of r-h-CuZnSOD and r-h-MnSOD in the peripheral blood were measured before and after their administration in dogs in groups 1A and 2.
The concentrations of both r-h-CuZnSOD and r-h-MnSOD peaked 10 minutes after administration; r-h-CuZnSOD remained relatively constant for 3 hours, and r-h-MnSOD remained at a level higher than r-h-CuZnSOD (Fig 10). The concentration of r-h-MnSOD was approximately 3 times higher than that of r-h-CuZnSOD after r-h-MnSOD was given as an intravenous bolus of 2 mg/kg and r-h-CuZnSOD was given as an intravenous bolus of 2 mg/kg and a continuous infusion at 0.8 mg/ (kg · h) (Fig 10).

Effects of r-h-CuZnSOD, r-h-MnSOD, Catalase, and Ketanserin on Ex Vivo Platelet Aggregation

The ex vivo platelet aggregation induced by ADP, thromboxane mimetic U46619, or serotonin was not significantly changed by treatment with r-h-CuZnSOD, r-h-MnSOD, or catalase (Fig 11). Platelet aggregation induced by platelet activating factor, however, was significantly inhibited by r-h-CuZnSOD, r-h-MnSOD, and catalase. Treatment with ketanserin inhibited serotonin-induced platelet aggregation but did not affect platelet aggregation induced by ADP, thromboxane mimetic, or platelet-activating factor.

Discussion

The present study demonstrates that administering the active oxygen species scavengers r-h-CuZnSOD, r-h-MnSOD, and catalase in vivo often eliminates platelet aggregation–associated cyclic flow reductions in severely stenosed and endothelium-injured canine coronary arteries.

Cyclic flow variations have been found to occur in the coronary arteries of some patients with unstable angina before and after coronary angioplasty.37 As originally described by Folts et al18 and further studied in our laboratory,26,30 cyclic flow reductions are caused by recurrent platelet aggregation and dislodgement on the endothelium-injured surface of a stenosed artery. Platelet-derived factors, such as thromboxane A2, serotonin, and ADP, are important mediators of cyclic flow variations.31,36,39 Inhibiting the effects of platelet-derived factors may eliminate cyclic flow variations.31,36,39 In the present study, r-h-CuZnSOD, r-h-MnSOD, and catalase abolished flow variations in up to 64% of the animals. Our data suggest that active oxygen species may be involved in mediating cyclic flow variations. This concept is also supported by the evidence that direct infusion of xanthine/xanthine oxidase (to generate superoxide anions) and hydrogen peroxide into the coronary artery induced cyclic flow variations.

By incubating xanthine/xanthine oxidase with platelets, Handin et al19 found that superoxide anions but not hydrogen peroxide enhance platelet aggregation and release reaction. However, other investigators have reported that hydrogen peroxide and hydroxyl radicals can cause and promote platelet aggregation and release reaction.15,25,25 In the present study, both superoxide dismutase and catalase significantly diminished platelet aggregation–associated cyclic flow variations in stenosed coronary arteries. In dogs in which r-h-MnSOD failed to eliminate cyclic flow variations, the addition of catalase did eliminate them. In dogs in which catalase failed to eliminate cyclic flow variations, however, the addition of r-h-MnSOD only worsened the cyclic flow variations. Also, both the incidence and frequency of cyclic flow variations induced by hydrogen peroxide were higher than those produced by xanthine/xanthine oxidase, the generator of superoxide anions. Our data suggest that hydrogen peroxide might be more important in mediating cyclic flow variations in this experimental animal model.

Fig 11. Graphs show ex vivo platelet aggregation before (control) and after different treatments. CuZnSOD indicates recombinant human copper-zinc superoxide dismutase; MnSOD, recombinant human manganese superoxide dismutase; U46619, a thromboxane mimetic; and PAF, platelet-activating factor. *P < .05 compared with control.
The mechanisms involved in activation of platelets by active oxygen species are unclear. Previous studies have shown that aspirin and indomethacin have no effect on superoxide anion–induced platelet release reaction, which suggests that thromboxane is not involved in the activation of platelets by active oxygen species.19 In the present study, r-h-CuZnSOD, r-h-MnSOD, and catalase significantly inhibited ex vivo platelet aggregation induced by platelet-activating factor but not by ADP, thromboxane mimetic, or serotonin. As shown in previous studies, platelet-activating factor is an important mediator of platelet aggregation and cyclic flow variations.40,41 Our data suggest that active oxygen species may affect the activation of platelets by platelet-activating factor.

Mehta et al42 and other investigators43-46 have reported that production of active oxygen species during reperfusion of the ischemic myocardium causes vessel dysfunction and that superoxide dismutase protects the vasodilator ability of the vessel. Gryglewski et al47 reported that superoxide anions are involved in the breakdown of endothelium-derived relaxing factor. Muge et al48 found that the release of intact endothelium-derived relaxing factor depends on endothelial superoxide dismutase activity. It also has been demonstrated that CuZnSOD enhances vasodilation in human coronary arteries mediated by endothelium-derived relaxing factor (personal communication, Dr Amnon Gonenne, Biotechnology, New York, NY). Studies from our laboratory indicate that endogenously produced endothelium-derived relaxing factor protects against platelet aggregation and cyclic flow variations.49 These findings suggest that active oxygen species may mediate cyclic flow variations by reducing the effect of endothelium-derived relaxing factor.

The effect of hydrogen peroxide on endothelium-derived relaxing factor is controversial. Kontos et al50 found that application of hydrogen peroxide to the surface of cat brain inhibits vasodilation mediated by endothelium-derived relaxing factor. Marczin et al51 discovered that treating calf pulmonary artery endothelial cells with hydrogen peroxide reduces the production of endothelium-derived relaxing factor. Burke-Wolin et al,52 however, demonstrated that hydrogen peroxide enhances the effect of endothelium-derived relaxing factor by activating guanylate cyclase, which results in the accumulation of cGMP. The effect of hydrogen peroxide on endothelium-derived relaxing factor and the contribution of hydrogen peroxide to cyclic flow variations in this model need further investigation.

Active oxygen species may alter human vascular endothelial cell function and may increase platelet adherence to endothelial cells.15 Hladovec52 reported that active oxygen species may cause endothelia mia in the rat. Poelstra et al53 demonstrated that active oxygen species can impair the vascular activity of ADPase. Our previous studies have shown that ADPase may prevent platelet aggregation and cyclic flow variations.31 Together, these data suggest that free radicals may contribute to the development of cyclic flow variations by damaging endothelial cells. We have provided further evidence of endothelial injury caused by active oxygen species in our scanning electron micrographs of coronary arteries exposed to xanthine/xanthine oxidase and hydrogen peroxide.

Epinephrine is present in the circulation but usually not in levels sufficiently high to activate platelets.54 In some situations, however, the concentration of epinephrine in the circulation increases dramatically. This increase could induce platelet aggregation even when the concentration of other platelet agonists is very low.55 Studies from our laboratory have revealed that, at certain doses, epinephrine was able to restore cyclic flow variations that had been eliminated by antagonists to certain platelet receptors, thromboxane, serotonin, and ADP.31,33,56 In the present study, epinephrine restored cyclic flow variations in most dogs treated singly with r-h-CuZnSOD, r-h-MnSOD, and catalase in a dose range similar to that in dogs treated with a single antagonist of ADP, thromboxane, and serotonin.31,33,56 Epinephrine restored cyclic flow variations in fewer dogs when the serotonin receptor antagonist, ketanserin, was added to the regimen. The dose of epinephrine required to restore cyclic flow variations was significantly higher in animals treated with the combination of ketanserin and active oxygen species scavengers than those treated with a single agent. These data suggest that, together, eliminating active oxygen species and blocking platelet receptors to serotonin may protect against the restoration of cyclic flow variations induced by epinephrine. Active oxygen species may have an additive effect with other platelet-derived factors on the activation of platelets. Protection against epinephrine-restored cyclic flow variation was slightly better in animals treated with a combination of r-hMnSOD and ketanserin than in those treated with r-h-CuZnSOD and ketanserin, perhaps because r-hMnSOD has a longer half-life in the circulation than r-h-CuZnSOD.57,58

In conclusion, active oxygen species may contribute to platelet aggregation and cyclic flow variations in severely stenosed and endothelium-injured canine coronary arteries through several different mechanisms. Eliminating active oxygen species in the circulation may prevent cyclic flow variations. Furthermore, eliminating active oxygen species and blocking platelet receptors to serotonin may protect against epinephrine-induced cyclic flow variations.

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