Nafazatrom-Induced Salvage of Ischemic Myocardium in Anesthetized Dogs is Mediated through Inhibition of Neutrophil Function

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SUMMARY. The effects of nafazatrom on leukocyte function in vitro and in vivo were related to its ability to salvage ischemic myocardium in an occlusion-reperfusion model of myocardial injury in the anesthetized dog. Nafazatrom (0.4–75 μM) produced dose-related inhibition in vitro of neutrophil aggregation, superoxide anion generation, arachidonic acid metabolism, and, to a lesser extent, the release of β-glucuronidase. In contrast, nafazatrom (0.4–37.5 μM) did not substantially influence platelet aggregation or the platelet metabolism of arachidonic acid. In vivo nafazatrom (10 mg/kg, po) reduced infarct size from 58 ± 3% of the risk area (mean ± SEM, n = 9) in control dogs to 23 ± 2% of the risk area (n = 9, P < 0.01). Nafazatrom also reduced the incidence of accompanying arrhythmias. Nafazatrom-induced myocardial salvage was not associated with any hemodynamic changes; moreover, it was independent of platelets, since thrombocytopenia did not prevent nafazatrom from exerting a protective effect. Measurements of the neutrophil-specific myeloperoxidase enzyme in ischemic myocardium indicate that the smaller infarct size in dogs treated with nafazatrom is accompanied by diminished leukocyte infiltration. Thus, the ability of nafazatrom to inhibit neutrophil function in vitro and cell infiltration in vivo may underly its myocardial-protective effects. (Circ Res 57: 131–141 1985)

ISCHEMIA-INDUCED myocardial damage is a dynamic process accompanied by the rapid accumulation of platelets (Leinberger et al., 1979; Laws et al., 1983) and neutrophils (Mullane et al., 1984) into the ischemic area. It has been suggested that platelet accumulation contributes to myocardial damage, since platelet microaggregates extend the area of infarct (Ruf et al., 1980) and induce fatal ventricular arrhythmias (Haeram, 1978). In addition, aggregating platelets release a variety of vasoactive mediators including thromboxane A2 (TxA2) and 5-hydroxytryptamine (5-HT), which induce coronary vasoconstriction (Mullane et al., 1982; De Clerck and Van Nueten, 1983).

Neutrophils, like platelets, can exacerbate ischemia-induced myocardial injury either by blocking coronary vessels to produce myocardial perfusion (Engler et al., 1983; Mullane et al., 1984) or through the elaboration of mediators. Stimulated neutrophils generate superoxide anions (Babior et al., 1977), lipooxygenase products of arachidonic acid (AA) (Borgeat and Samuelsson, 1979), and platelet-activating factor (Clark et al., 1980), and release various proteolytic enzymes (Zurier et al., 1974), all of which could enhance tissue injury and the extent of the myocardial damage (Mullane and Moncada, 1983). This hypothesis is supported by the finding that drugs directed against the neutrophils to prevent their activation and infiltration (Mullane and Moncada, 1982; Romson et al., 1982; Mullane et al., 1984; Jolly et al., 1984), or which induce neutropenia (Romson et al., 1983; Mullane et al., 1984), also reduce infarct size.

Nafazatrom (Bay g 6575) is a new antithrombotic agent (Seuter et al., 1979) which reduces ischemic damage in the myocardium of rats (Fiedler, 1983a) and rabbits (Fiedler, 1984). Moreover, nafazatrom inhibits the metabolism of AA via the lipooxygenase enzyme in a melanoma cell line (Honn and Dunn, 1982). We, and others, have previously demonstrated that BW755C, which inhibits the lipooxygenase and cyclooxygenase pathways of AA metabolism, reduces infarct size, whereas cyclooxygenase inhibitors do not (Mullane and Moncada, 1982; Jolly and Lucchesi, 1983; Mullane et al., 1984). The reduction in ischemic damage by BW755C correlates with suppression of neutrophil function (Mullane et al., 1984). Thus, if nafazatrom also inhibits the neutrophil lipooxygenase enzyme, a dual action on neutrophils and platelets may make nafazatrom an ideal agent for the salvage of ischemic myocardium.

The initial aim of this study was to assess the ability of nafazatrom to influence leukocyte and platelet behavior in vitro, viz., cell aggregation and AA metabolism, together with neutrophil superoxide anion generation and release of lysosomal enzymes. In these experiments, nafazatrom was compared with BW755C. The second aim of this study was to determine whether nafazatrom could salvage ischemic myocardium in an occlusion-reperfusion model of myocardial injury in the chloralose-anesthetized dog, and to determine how the degree
of damage correlated with an effect on platelet and/or neutrophil activity in vitro.

Methods

The effects of nafazatrom on leukocyte function, viz., cell aggregation, release of 3-glucuronidase and superoxide anions, and metabolism of [14C]arachidonic acid were investigated and compared with BW755C, a known inhibitor of leukocyte infiltration into inflammatory lesions (Higgs et al., 1980). The activity of nafazatrom on neutrophils was contrasted with its effects on platelet aggregation and platelet metabolism of [14C]arachidonic acid. Initially, studies were performed on rat neutrophils because they are an inexpensive and readily available source of large numbers of cells, and the results were subsequently confirmed in cells obtained from the dog.

Neutrophil Harvesting

Peritoneal neutrophils were harvested after the instillation of either 12 ml of 8% casein (rat) or 400 ml of 0.1% glycogen (dog) into the peritoneal cavity for 12 or 3 hours, respectively. Contaminant red blood cells in the aspirated peritoneal fluid were destroyed by hypotonic lysis. The remaining leukocytes were washed in normal saline and resuspended at 1 x 10^7/ml in Hank's balanced salt solution, pH 7.4. Wright-Giemsa staining showed that leukocyte suspensions from both species contained 85-95% neutrophils with greater than 95% viability, as assessed by trypan blue exclusion.

Platelet Harvesting

Washed platelets were prepared according to the method of Vargas et al. (1982). Briefly, blood was collected into 3.8% sodium citrate (9:1, vol/vol) containing 2 µg/ml prostaglandin I2 ([PGI2] Upjohn Co.; prepared fresh as a 1 mg/ml stock solution in 1 M Tris buffer, pH 8.5). The platelets then were isolated and washed in Tyrode's buffer [(PGI2) Upjohn Co.; prepared fresh as a 1 mg/ml stock solution in 1 M Tris buffer, pH 8.5) and resuspended at 10^7/ml in Hank's balanced salt solution, pH 7.4. Wright-Giemsa staining showed that leukocyte suspensions from both species contained 85-95% neutrophils with greater than 95% viability, as assessed by trypan blue exclusion.

Platelet and Neutrophil Aggregation

Platelet aggregation induced by adenosine diphosphate (ADP) 10 µM, prepared in 0.9% saline, pH 7.4; Sigma Chemical Co.) was studied with a Payton light aggregometer. A platelet suspension (0.5 ml) containing 2 x 10^8 platelets/ml was added to a siliconized glass cuvette and the generation of superoxide anions (O2-) was measured spectrophotometrically as the reduction of cytochrome c (Babior et al., 1973). Briefly, 400 µl of a neutrophil suspension (1.5 x 10^7/ml) was preincubated with cytochalasin B (3.75 µg Sigma Chemical Co.) for 15 minutes at 37°C in the presence or absence of nafazatrom or BW755C, followed by the addition of ferricytochrome c (horse heart type III, Sigma; 0.57 mg). A duplicate set of samples contained 25 µg superoxide dismutase. The incubates were stimulated with 10^-7 M FMLP or 1.9 x 10^-7 M A23187, and the reaction was stopped after 20 minutes by the addition of excess superoxide dismutase. The superoxide dismutase-sensitive reduction of ferricytochrome c was measured spectrophotometrically (550 nm, Beckman spectrophotometer, model 260).

β-Glucuronidase Assay

Neutrophils (400 µl; 1.5 x 10^7/ml) were preincubated with cytochalasin B (3.75 µg) in the presence or absence of BW755C, nafazatrom, or the appropriate vehicle. The cells were then incubated for 20 minutes at 37°C with FMLP (10^-7 M). After being subjected to high speed centrifugation (10,000 g, 30 seconds) to terminate the reaction, the cell-free supernatant extract containing the released enzyme was incubated in the presence of phenolphthalein mono-β-glucuronic acid. β-Glucuronidase liberates free phenolphthalein, measured spectrophotometrically at 550 nm (Fishman et al., 1967), where one unit of enzyme activity is that which liberates 1 µg phenolphthalein per hour at 56°C.

Animal Preparation

Male dogs, 15–20 kg, were anesthetized initially with Biotal (thiomylal sodium, Bio-Ceutic), and anesthesia was maintained with α-chloralose (80 mg/kg, Koch-Light Laboratories). The dogs were intubated and artificially ventilated with room air. The femoral artery and vein were cannulated for the measurement of blood pressure and
for drug administration, respectively. ECG was monitored from limb lead II.

The heart was exposed by a thoracotomy at the 5th intercostal space and suspended in the pericardial cradle. Left ventricular pressure and its first derivative (dP/dt) were measured via a micromanometer-tipped catheter (Gaeltec, 7F) inserted through a stab wound in the apex of the heart and anchored with purse-string suture. A segment of the left anterior descending (LAD) coronary artery was dissected free at a point just below its first major diagonal branch (Fig. 1). A screw clamp and an occlusive snare ligature were placed around this segment. Coronary blood flow was measured from an electromagnetic flow probe (Carolina Medical Electronics) placed distal to the screw clamp. The coronary artery was occluded for 90 minutes followed by 5 hours of reperfusion.

Incidence of Arrhythmias

The number of premature ventricular contractions (PVC) during the 90-minute period of occlusion and the first 30 minutes of reperfusion (which are the two most vulnerable periods for ventricular fibrillation to occur) was recorded. The incidence of arrhythmias in control and drug-treated dogs was compared by Wilcoxon-Mann-Whitney test for nonparametric data, while the number of animals undergoing ventricular fibrillation was compared by \( \chi^2 \) test.

Quantification of the Area at Risk and Infarct Size

Those portions of the myocardium at risk of becoming infarcted, and which were infarcted, were delineated by a dual-staining technique adapted from the method of Warrtier et al. (1981), which we have described previously (Mullane and Moncada, 1982; Mullane et al., 1984; see Fig. 1). At the end of the 5-hour reperfusion period, 50 ml of whole blood were collected into a tube containing the heparin (10 units/ml final concentration.) The previously occluded segment of the LAD coronary artery was cannulated and the heparinized blood was reinfused at mean systemic blood pressure. Simultaneously, 20 ml of Evans' blue dye (Sigma Chemical Co.) were injected into the coronary bed so that area which was perfused with the undyed blood. The dog was killed, and its heart was removed and sliced (0.5 cm thick) in breadloaf fashion from apex to base. The area free of the blue dye indicates that portion of the left ventricle served by the occluded segment of the LAD, called the risk area. The slices of myocardium then were incubated with triphenyltetrazolium dye (TPT) in phosphate-buffered saline, pH 7.4, for 20 minutes (Jestaadt and Sandritter, 1959). Whereas the viable tissue stains brick red, the infarcted area remains colorless. The upper surface of each slice and the boundaries of the infarct zone, risk area, and tissue were traced onto a clear acetate sheet and the areas of each zone were measured by planimetry. Infarct size, expressed as a percentage of the area at risk, was compared in drug-treated and control animals by one-way analysis of variance.

Myeloperoxidase Assay

The neutrophil-specific myeloperoxidase (MPO) enzyme was measured by an adaptation of the method described by Bradley et al. (1982). Cardiac tissue taken from normal and infarcted regions of the heart were sequentially frozen in liquid nitrogen, pulverized, suspended as a homogenate in 50 mM potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide to solubilize the MPO enzyme, blended in a Waring blender for 1–5 minutes, and further disrupted with 4 strokes of an Oxwell 3000 mechanical mortar and pestle. After being sonically disrupted for 10 seconds (Fisher Sonic Dismembrator, model 300), the homogenate was freeze-thawed 3 times with liquid nitrogen and sonicated for 10 more seconds. The supernatant which contains the MPO enzyme (Bradley et al., 1982) was separated from the cell debris by centrifugation at 40,000 g for 15 minutes.

MPO activity was assayed in duplicate using dianisidine-hydrogen peroxide as substrate. One-tenth milliliter of the supernatant was mixed with 2.9 ml of 50 mM phosphate buffer (pH 6.0) containing 0.167 ng/ml O-dianisidine hydrochloride and 0.0005% hydrogen peroxide, and the change in absorbance at 460 nm was measured on a Beckman model 26 spectrophotometer. The results were expressed as units (U) of MPO/100 mg tissue (wet weight), where one unit of MPO activity is defined as that degrading 1 μmol of peroxide per minute at 25°C (Bradley et al., 1982). We similarly treated samples of canine neutrophils to assess the relationship between MPO activity and tissue neutrophil content.

Histological Sections

Sections of myocardium were prepared for histological verification of neutrophil infiltration as previously described (Mullane et al., 1984). The tissue was placed in neutral-buffered formalin, routinely processed for paraffin histology, and stained with hematoxylin and eosin.

Preparation of Anti-Platelet Serum

Anti-dog platelet antiserum was a generous gift of Dr. S. Kunkel, University of Michigan. Canine platelets, isolated by gel filtration chromatography using sepharose 2B equilibrated with a calcium-free phosphate buffer (100 mM, pH 7.0) were mixed with incomplete Freund's adjuvant and injected intradermally into a sheep. Twenty-five days later, the sheep was bled, and the serum was harvested and heat-inactivated.
Experimental Protocol for in Vivo Studies

In studies on infarct size, four groups of dogs were used. The first group consisted of 17 control animals. The second group contained 10 dogs which were treated orally with 10 mg/kg nafazatrom contained in a small portion of dog food. The drug was administered 1.5–2 hours before induction of anesthesia, approximately 4 hours before the coronary artery was occluded. In group 3, seven dogs were given nafazatrom, 1.5 mg/kg, 30 minutes before coronary occlusion, and a second dose of 0.5 mg/kg was given 30 minutes after reperfusion. These doses of nafazatrom were chosen from previous studies in which oral pretreatment at 5 or 10 mg/kg reduced infarct size and canine coronary artery thrombosis (Fiedler, 1983b; 1984), as did pretreatment with 1 mg/kg, iv (Shea et al., 1984a). The fourth group of six dogs were given nafazatrom 10 mg/kg, po, in addition to sheep anti-dog platelet antiserum to deplete circulating platelets, in a dose of 0.25 ml serum/kg. Platelet counts (1:1000 dilution of platelet-rich plasma in 0.9% vol/vol saline) were determined before and 30 minutes after administration of the antiserum, and periodically thereafter. After surgical preparation, the animals were allowed to equilibrate for 30 minutes, during which time the screw clamp on the LAD coronary artery was advanced so as to reduce the reactive hyperemia following a 10-second occlusion by more than 70%, without reducing mean coronary blood flow. [Reperfusion into a critical stenosis reduces the incidence of hemorrhagic infarction and ventricular fibrillation upon reperfusion (Lucchesi et al., 1976).] Any animals which fibrillated during this period were excluded from the study. After the heart had been stained to demarcate the infarcted zone and the risk area, samples of this myocardium were taken and frozen in liquid nitrogen for the MPO assay.

Results

Rat Neutrophil Function in Vitro

Neutrophil Aggregation

The addition of \(10^{-7}\) to \(10^{-5}\) M FMLP to suspensions of rat neutrophils resulted in dose-dependent aggregation which peaked within 3 minutes. With the difference in light transmission between neutrophil suspensions containing 100% and 80% cells set at 10 arbitrary units, \(10^{-6}\) M FMLP produced 10–12 units of aggregation, whereas \(10^{-5}\) M FMLP induced an aggregation of 7 ± 0.5 units, and was used as the submaximal stimulus. Preincubation with nafazatrom (0.4–37.5 \(\mu\)M) resulted in dose-related inhibition of aggregation from 10 ± 2% to 68 ± 7% with a submaximal stimulus. Preincubation with nafazatrom and BW755C in a dose-dependent manner by nafazatrom (IC\(_{50} = 4.7 \mu\text{M}, n = 5)\) or BW755C (IC\(_{50} = 42.6 \mu\text{M}, n = 5\); Table 1).

Lysoosomal Enzyme Release

The addition of \(10^{-7}\) M FMLP to incubates of rat neutrophils resulted in the release of 81 ± 9 units of \(\beta\)-glucuronidase activity. Significant inhibition of enzyme release by nafazatrom was obtained only at the highest drug concentrations used, where 37.5 and 75 \(\mu\)M yielded 15 ± 2% and 24 ± 2% inhibition, respectively (both \(P < 0.05, n = 5\)). BW755C, at concentrations up to 94 \(\mu\)M, did not significantly affect the release of \(\beta\)-glucuronidase.

Metabolism of \([^{14}C]\)Arachidonic Acid

Incubation of neutrophils with \([^{14}C]\)AA for 10 minutes results in the formation of cyclooxygenase and lipoxygenase products (Fig. 3). In rat neutrophils, preincubation with 75 \(\mu\)M nafazatrom reduced the formation of lipoxygenase products by 51 ± 11% while increasing cyclooxygenase products by 24 ±

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td><strong>FMLP-Induced Superoxide Anion Generation by Rat Neutrophils is Inhibited by Nafazatrom and BW755C</strong></td>
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</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Nafazatrom</td>
</tr>
<tr>
<td>1.8 (\mu)M</td>
</tr>
<tr>
<td>7.5 (\mu)M</td>
</tr>
<tr>
<td>37.5 (\mu)M</td>
</tr>
<tr>
<td>BW755C</td>
</tr>
<tr>
<td>3.8 (\mu)M</td>
</tr>
<tr>
<td>37.5 (\mu)M</td>
</tr>
<tr>
<td>188.0 (\mu)M</td>
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</table>

NS = not significant.
FIGURE 3. Effects of nafazatrom on the metabolism of [14C]arachidonic acid (AA) by rat neutrophils. Products were extracted and separated by thin-layer chromatography and visualized by autoradiography. The migration of authentic standards is shown on the left, and the percent radioactivity of each region is shown on the right.

16% (n = 5), suggesting that nafazatrom was acting as a selective inhibitor of the lipoxygenase enzyme.

Canine Neutrophil Function in Vitro

Neutrophil Aggregation

Dog neutrophils aggregated 9.8 ± 1.1 units upon the addition of 9.5 × 10^{-8} M A23187 (n = 5). Nafazatrom inhibited A23187-induced aggregation, with an IC_{50} of approximately 18 μM. In contrast to rat cells, canine neutrophils do not aggregate to FMLP.

Superoxide Anion Formation

The incubation of A23187 (9.5 × 10^{-8} M) with canine neutrophils resulted in the formation of 7.8 ± 1.4 nmol O_2 at 20 minutes. Nafazatrom, 7.5–37.0 μM, produced dose-related inhibition of superoxide formation of 50 ± 20% to 86 ± 7% with an IC_{50} of 7.5 μM (n = 3).

Metabolism of [14C]Arachidonic Acid

Pretreatment of dog neutrophils with nafazatrom before adding [14C]AA resulted in potent inhibition of the lipoxygenase pathway, with inhibition ranging from +9 ± 20 to −70 ± 9% at concentrations of 1.8–37.5 μM (IC_{50} = 7.1 μM, Table 2). In contrast to the rat neutrophils, where nafazatrom appears specific for the lipoxygenase enzyme, in canine neutrophils nafazatrom was also an equipotent inhibitor of prostaglandin production, with suppression ranging from +1 ± 12 to 70 ± 7% at concentrations of 1.8–37.5 μM (IC_{50} = 4 μM). Higher concentrations of nafazatrom produced no further inhibition of either pathway in these cells.

Inhibition of the cyclooxygenase pathway by nafazatrom appeared to be specific for dog neutrophils, since in addition to the rat neither rabbit nor human neutrophils showed a diminution of prostaglandin production after preincubation with nafazatrom (data not shown). Pretreatment of dog neutrophils with BW755C resulted in a dose-related inhibition of both cyclooxygenase and lipoxygenase products (Table 2) with IC_{50} calculated to be 25.5 and 22.1 μM, respectively. These inhibitors did not influence the recovery of radiolabeled material. The amount of radioactivity recovered from control incubates was 91,152 ± 13,606 counts/min, and from nafazatrom-treated incubates was 70,567 ± 10,117 counts/min. In experiments with BW755C, the values were 77,495 ± 19,236 and 70,149 ± 21,210 counts/min for control and drug-treated incubates, respectively.

In homogenates of canine neutrophils, nafazatrom was a less potent inhibitor of the lipoxygenase enzyme, where 75 μM produced 50 ± 4% inhibition. In these broken cells, nafazatrom did not inhibit the cyclooxygenase enzyme, where cyclooxygenase products accounted for 16 ± 5% vs. 19 ± 6% of the radioactivity recovered for control and nafazatrom-treated incubates, respectively (n = 3). The results obtained in canine neutrophil homogenates are very similar to those obtained in intact rat neutrophils.

Canine Platelet Function in Vitro

In contrast to the effects on neutrophil aggregation, pretreatment of washed platelets with nafazatrom at concentrations up to 10 μM did not significantly affect ADP-induced platelet aggregation, whereas the same concentration of BW755C produced an 80% reduction in platelet aggregation. Higher doses of nafazatrom, up to 37.5 μM, induced a small (<32%) inhibition of the aggregatory response.

The incubation of [14C]AA with washed platelets led to the formation of 12-hydroxyeicosatetraenoic acid (12-HETE), thromboxane B_2 (TxB_2), and hydroxy heptadecatrienoic acid (HHT) as the major products. At concentrations up to 75 μM, nafazatrom did not alter the profile of AA metabolism, with

### Table 2

<table>
<thead>
<tr>
<th>Nafazatrom (μM)</th>
<th>BW755C (μM)</th>
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<tbody>
<tr>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>7.5</td>
<td>7.5</td>
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<tr>
<td>37.5</td>
<td>94.0</td>
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<table>
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<tr>
<th>Metabolism</th>
<th>Nafazatrom (μM)</th>
<th>BW755C (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclooxygenase</td>
<td>+1.1 ± 12.4</td>
<td>−58.6 ± 6.4</td>
</tr>
<tr>
<td></td>
<td>−70.1 ± 7.0</td>
<td>−28.6 ± 8.8</td>
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<tr>
<td></td>
<td>−65.4 ± 4.6</td>
<td>−77.8 ± 4.8</td>
</tr>
<tr>
<td>Lipoxygenase</td>
<td>+9.0 ± 20.3</td>
<td>−53.8 ± 4.9</td>
</tr>
<tr>
<td></td>
<td>−69.6 ± 8.6</td>
<td>−33.2 ± 11.8</td>
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<tr>
<td></td>
<td>−61.9 ± 5.0</td>
<td>−81.3 ± 5.5</td>
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</table>

Results are expressed as the percentage change from the control values (mean ± SEM; n = 3–5). Cyclooxygenase and lipoxygenase zones are as defined in Methods.
cyclooxygenase and lipoxygenase products representing 116 ± 6% and 97 ± 9% of control values (Fig. 4). BW755C (94 μM) reduced the formation of lipoxygenase and cyclooxygenase products by 94 ± 1% and 88 ± 5%, respectively. Neither nafazatrom nor BW755C altered the recovery of 14C-labeled products. Moreover, nafazatrom was also inactive against the cyclooxygenase and lipoxygenase enzymes in an experiment using platelet homogenates. In these broken platelets, cyclooxygenase products accounted for 36% of the recovered radioactivity in control incubates, and for 37% after nafazatrom treatment (75 μM). Lipoxygenase products accounted for approximately 23% and 25% of the recovered radioactivity in control and nafazatrom-treated homogenates, respectively.

Effects of Nafazatrom on Infarct Size

Forty dogs were subjected to coronary occlusion and reperfusion, of which eight fibrillated and were excluded from the study. Control dogs (n = 9) had a risk area comprising 33 ± 4% left ventricle, of which 58 ± 3% became infarcted. Dogs pretreated with nafazatrom (10 mg/kg, po, n = 9) had a similar risk area of 31 ± 4% of left ventricle; however, only 23 ± 2% of this risk area became infarcted (P < 0.01, Fig. 5). In contrast, dogs treated with nafazatrom, iv (1.5 mg/kg + 0.5 mg/kg) showed no reduction in infarct size (49 ± 10% risk area where the risk area = 34 ± 1% left ventricle).

The myocardial protection afforded by oral nafazatrom appeared to be independent of any hemodynamic effects, since nafazatrom was devoid of any demonstrable cardiovascular action whether given by the oral or intravenous route (Table 3). Moreover, oral nafazatrom did not alter the hemodynamic changes produced by coronary occlusion and reperfusion (Table 4; Fig. 6). For example, the rate-pressure product, considered by some to be an index of cardiac work (Gobel et al., 1978), exhibited the same temporal changes throughout the experiment in both control and nafazatrom-treated dogs (Fig. 6).

Platelet Depletion, Neutrophil Infiltration, and Nafazatrom

The contribution of platelets to the myocardial protective effects of nafazatrom was investigated in a group of dogs rendered thrombocytopenic with specific anti-platelet antiserum. We have previously demonstrated that this antiserum reduces circulating platelets by >90% without reducing infarct size (Mullane and McGiff, in press). (The results of the previous study on infarct size in thrombocytopenic dogs is included in Figure 5 for clarity.) In the

![Figure 4](image_url)

**Figure 4.** Effects of nafazatrom on the metabolism of [14C]arachidonic acid (AA) by washed dog platelets. For explanation, see Figure 3.

**Figure 5.** Nafazatrom-induced myocardial salvage. Each symbol represents an individual experiment, adjacent to which is the mean ± SEM for each group. APAS = anti-platelet antiserum.

**Table 3**

<table>
<thead>
<tr>
<th></th>
<th>Mean systemic blood pressure (mm Hg)</th>
<th>Heart rate (beats/min)</th>
<th>Left ventricular pressure (mm Hg)</th>
<th>Positive dP/dt max (mm Hg/sec)</th>
<th>Negative dP/dt max (mm Hg/sec)</th>
<th>LAD coronary flow (ml/min)</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Nafazatrom (10 mg/kg, po)</td>
<td>129 ± 11</td>
<td>145 ± 8</td>
<td>128 ± 5</td>
<td>2450 ± 319</td>
<td>2225 ± 223</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>Nafazatrom (1.5 mg/kg, iv)</td>
<td>133 ± 5</td>
<td>136 ± 8</td>
<td>129 ± 2</td>
<td>2320 ± 110</td>
<td>2370 ± 120</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>APAS + nafazatrom (10 mg/kg, po)</td>
<td>137 ± 6</td>
<td>144 ± 7</td>
<td>137 ± 2</td>
<td>2660 ± 130</td>
<td>2642 ± 104</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>APAS</td>
<td>125 ± 4</td>
<td>154 ± 6</td>
<td>141 ± 2</td>
<td>2571 ± 286</td>
<td>2635 ± 327</td>
<td>22 ± 3</td>
</tr>
</tbody>
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Values represent mean ± SEM; n = number of dogs. APAS = anti-platelet antiserum.
The Influence of Drug Treatment on the Temporal Changes in Mean Blood Pressure (±SEM) during Occlusion and Reperfusion

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>Pre-Occlusion</th>
<th>Occlusion</th>
<th>Reperfusion</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>15'</td>
<td>30'</td>
<td>60'</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>129±11</td>
<td>114±11</td>
</tr>
<tr>
<td>Nafazatrom 10 mg/kg, po</td>
<td>9</td>
<td>133±5</td>
<td>128±7</td>
</tr>
<tr>
<td>Anti-platelet serum</td>
<td>6</td>
<td>125±4</td>
<td>117±7</td>
</tr>
<tr>
<td>Anti-platelet serum plus nafazatrom</td>
<td>6</td>
<td>131±6</td>
<td>136±7</td>
</tr>
</tbody>
</table>

In the present study, six dogs treated with anti-platelet antisemum showed a reduction in platelet numbers from 276 ± 15 x 10^9/ml to 17 ± 1 x 10^9/ml (P < 0.001, paired t-test). Infarct size in thrombocytopenic dogs treated with nafazatrom (10 mg/kg, po) was 24 ± 3% risk area, which was significantly less than that observed in control or platelet-depleted animals [both P < 0.01 (Fig. 6)]. The size of the risk area, 34 ± 1.5% left ventricle, was not significantly different. Platelet-depleted animals given nafazatrom showed no significant hemodynamic changes (Tables 3 and 4; Fig. 6). Although there was a tendency for blood pressure to be better maintained in these animals, in particular at the later stages of reperfusion, there were no significant differences in blood pressure (P = 0.092, ANOVA).

We have recently adapted an assay for the neutrophil-specific MPO enzyme (Bradley et al., 1982) to assess neutrophil infiltration into the ischemic myocardium (Mullane, Kraemer, and Smith, unpublished observations). MPO activity in control regions of the heart is very low at 0.016 ± 0.005 U MPO/100 mg tissue. However, infarcted regions of the heart show a significant (P < 0.01) increase in MPO activity to 0.33 ± 0.03 U/100 mg tissue (n = 6). This amount of MPO activity is equivalent to that in 5.5 ± 0.5 x 10^5 neutrophils. In contrast, infarcted myocardium taken from dogs treated with nafazatrom showed a significant reduction in MPO activity (Fig. 7) to 0.034 ± 0.01 U/100 mg tissue (n = 8, P < 0.01 vs. infarcted myocardium of control dogs). This level of activity represents 3.0 ± 0.6 x 10^4 neutrophils. Nafazatrom did not directly inhibit the MPO enzyme, since the addition of nafazatrom (1-2 μg/ml) to infarcted myocardial incubates did not significantly reduce the MPO activity (0.042 ± 0.004 vs. 0.036 ± 0.003 U/100 mg tissue for control and nafazatrom-treated incubates, respectively, n = 3). Histological sections confirmed the lack of neutrophils in the ischemic regions of the myocardium of nafazatrom-treated dogs (Fig. 7).

Nafazatrom and Ventricular Arrhythmias

Ventricular fibrillation was observed in 41% of control dogs (7/17). Of these, three fibrillated within 15 minutes of occluding the coronary artery, while four fibrillated during the first 20-25 minutes of reperfusion. Reperfusion-induced ventricular fibrillation was not observed in dogs treated orally with nafazatrom, whereas one of 15 (6%) of these dogs fibrillated 2 minutes after coronary occlusion. None of the seven dogs treated with iv nafazatrom underwent ventricular fibrillation in either the occlusion or reperfusion period. Thus, nafazatrom significantly reduced the likelihood of ventricular fibrillation (P < 0.001 for both treatment groups, χ² test).

The effect of nafazatrom on the incidence of
premature ventricular contractions is shown in Table 5. Nafazatrom, whether given orally or intravenously, reduced the number of ectopic beats during the occlusion phase. There was also a reduction in PVC in the first 30 minutes of reperfusion in nafazatrom-treated dogs, but this was significant only in the orally treated group.

Discussion

Nafazatrom reduces infarct size in an occlusion-reperfusion model of myocardial injury in the chloroalose-anesthetized dog. Nafazatrom has also been shown to reduce infarct size in rats (Fiedler, 1983a), rabbits (Fiedler, 1984), and, recently, in dogs (Shea et al., 1984b). However, these studies did not address the mechanism whereby nafazatrom may exert this protective effect. There are a number of mechanisms which could account for the reduction in ischemic damage seen with this drug.

Recent studies have implicated the neutrophils as important contributors to ischemia-induced myocardial damage (see Mullane and Moncada, 1983 for review), since neutrophil infiltration is temporally related to the necrotic process (Mullane et al., 1984) and drugs directed against the neutrophils reduce infarct size (Mullane and Moncada, 1982; Romson et al., 1982, 1983; Mullane et al., 1984). Stimulation of neutrophils by FMLP, or complement fragments, the latter being formed during acute myocardial infarction (Hill and Ward, 1971; McManus et al., 1983), causes the cells to aggregate, adhere avidly to surfaces, migrate along a chemotactic gradient, and release pro-inflammatory mediators at a site of damage (Weissmann, 1982). Inflammatory mediators, including free radicals of oxygen (e.g., superoxide anions), lipoxygenase products of AA, and platelet-activating factor, are formed and released,
together with lysosomal enzymes such as \( \beta \)-glucoronidase. These inflammatory mediators can promote tissue damage and thus exacerbate ischemia-induced myocardial injury. The in vitro experiments indicate that nafazatrom inhibits neutrophil aggregation (which has some analogies to chemotaxis, (Yuli and Synderman, 1984), attenuates the generation of superoxide anions, prevents the lipooxygenation of arachidonic acid, and produces a small reduction in the release of lysosomal enzymes. Thus, nafazatrom inhibits many different aspects of neutrophil function in vitro, although it is important to recognize that cells harvested from the peritoneum may not respond identically to the circulating neutrophils. Such a broad spectrum of actions may suggest that nafazatrom inhibits some very early process of neutrophil activation, such as the increase in intracellular calcium (Petroski et al., 1979) or the generation of cyclic adenosine monophosphate (cAMP) (Smolen et al., 1980), which precedes mediator release. An effect on intracellular calcium is unlikely, since platelet activation is also accompanied by a rise in intracellular calcium (Owen and Le Breton, 1981), and platelet behaviour in vitro was not affected by nafazatrom. Moreover, although it was anticipated that inhibition of an early event might cause similar decreases in lysosomal enzyme release, superoxide anion formation, and cell aggregation, this was not the case. Finally, nafazatrom inhibited the metabolism of exogenously administered AA in neutrophil homogenates (albeit at higher concentrations than those needed in intact cells), indicating that, in this instance at least, nafazatrom directly affected a specific enzyme within the neutrophil. BW755C, an inhibitor of AA metabolism by the lipoxygenase and cyclooxygenase enzymes (Higgs et al., 1979), has also been described as a free-radical scavenger (Duniec et al., 1983), shares many similarities with nafazatrom. BW755C inhibits neutrophil aggregation and formation of superoxide anions as well as AA metabolism, but did not inhibit the release of \( \beta \)-glucuronidase. Similar results were obtained with nafazatrom in canine neutrophils stimulated with the calcium ionophore, A23187, indicating that the inhibition of neutrophil function is not specific for FMLP or due to prevention of ligand-receptor binding. Suppression of neutrophil activation by two chemically dissimilar inhibitors of AA metabolism may support the hypothesis that a metabolite of AA is involved in neutrophil activation, perhaps by altering calcium mobilization (Naccache et al., 1981).

The ability of nafazatrom to inhibit neutrophil activation in vitro and reduce infarct size in vivo suggests that the two actions may be linked. To assess more accurately the degree of neutrophil infiltration into the myocardium, we have utilized a quantitative assay for the neutrophil-specific myeloperoxidase enzyme (Bradley et al., 1982). Infarcted myocardium from nafazatrom-treated dogs had significantly lower myeloperoxidase levels than tissue samples from control animals, indicating that nafazatrom suppressed neutrophil infiltration into the ischemic heart. Histological analyses confirmed the results of the myeloperoxidase assay. Therefore, nafazatrom inhibits leukocyte function in vitro and in vivo, and this action may well account for the myocardial salvage.

A second mechanism by which nafazatrom may salvage ischemic myocardium is via its anti thrombotic properties. Although nafazatrom did not influence aggregation or arachidonate metabolism in platelets in vitro, confirming previous results in other species (Seuter et al., 1979; Fischer et al., 1983), it is antithrombotic and thrombolytic in vivo (Seuter et al., 1979). However, platelet depletion with specific anti-platelet antisera does not reduce infarct size or the incidence of arrhythmias in this occlusion-reperfusion model (Mullane and McGiff, in press); neither does it prevent myocardial salvage induced by nafazatrom. Thus, the myocardial protective effect of nafazatrom appears independent of platelets.

The ability of nafazatrom to inhibit prostaglandin 15-hydroxydehydrogenase [PGDH (Wong et al., 1982)] may underly another potential myocardial protective mechanism. PGDH catalyzes the first step in prostaglandin metabolism. The potent coronary vasodilator PGI2 can be metabolized by this enzyme (Wong et al., 1978), and nafazatrom increases the release of PGI2 from vascular tissue (Vermeylen et al., 1979). Since PGI2 reduces infarct size (Ribeiro et al., 1981; Jugdutt et al., 1981; Melin and Becker, 1983), salvage of ischemic myocardium by nafazatrom could be mediated through an increase in PGI2. However, nafazatrom reduces infarct size in rats (Fiedler, 1983a), where PGI2 is ineffective (Johnson et al., 1983), suggesting that the myocardial protective effects of nafazatrom are independent of PGI2.

The lack of any apparent cardiovascular effect with nafazatrom indicates that the myocardial protective effects of this drug are not due to hemodynamic changes which improve the myocardial oxygen supply-and-demand ratio (Maroko et al., 1971). Although such a mechanism appears unlikely, changes in collateral blood flow to the ischemic region were not assessed, and could contribute to the myocardial salvage.

Nafazatrom reduced the incidence of premature ventricular contractions and fibrillation. An antiarrhythmic effect was also observed with BW755C (Mullane et al., 1984). The mechanism underlying this action is not known. It does not appear to be secondary to the myocardial salvage, since dogs treated with 1.5 mg/kg nafazatrom intravenously had significantly less arrhythmias, with no reduction in infarct size. Whether lipoxygenase products or other mediators released from activated leukocytes during ischemia promote electrical instability within the myocardium remains to be determined.

The failure of intravenously administered nafazatrom to reduce infarct size may relate to the phar-
macokinetics of the drug. Nafazatrom undergoes extensive biotransformation during first passage through the liver. The elimination half-life of radiolabeled nafazatrom is approximately 3-3.5 hours after intravenous administration, but 8.5 hours after oral dosing (Philipp et al., 1983). Consequently, adequate plasma levels of nafazatrom may not be sustained after intravenous administration to inhibit leukocyte infiltration into the ischemic myocardium effectively.

In summary, nafazatrom salvages ischemic myocardium in an occlusion-reperfusion model of myocardial injury in the anesthetized dog. This effect was independent of any hemodynamic or anti-thrombotic properties of the drug. Since nafazatrom potently inhibited different aspects of neutrophil function in vitro and suppressed neutrophil infiltration into ischemic myocardium in vivo, it is suggested that the myocardial protective effects of nafazatrom are mediated through inhibition of neutrophil activity. Moreover, this study provides the first evidence for a correlation between the activity of a drug on leukocyte function in vitro and its ability to salvage ischemic myocardium in vivo, and suggests that in vitro leukocyte assays may be a useful, simple, screening method for drugs capable of reducing infarct size by interfering with the inflammatory process.

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