Role of activated platelets in excitation of cardiac afferents during myocardial ischemia in cats

LIANG-WU FU AND JOHN C. LONGHURST
Department of Medicine, University of California, Irvine, California 92697-4075
Received 28 June 2001; accepted in final form 18 September 2001

Fu, Liang-Wu, and John C. Longhurst. Role of activated platelets in excitation of cardiac afferents during myocardial ischemia in cats. Am J Physiol Heart Circ Physiol 282: H100–H109, 2002.—Myocardial ischemia activates cardiac spinal afferents that mediate chest pain and excitatory cardiovascular responses. Platelets are activated dur-
ing myocardial ischemia and release 5-hydroxytryptamine, which stimulates abdominal spinal afferents. This study in-
vestigated the role of activated platelets in excitation of cardiac spinal afferents during ischemia. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained from cats and incubated with collagen (2 mg/ml) or thrombin (5 U/ml). We observed reduction of platelets in PRP indicative of platelet activation by collagen and thrombin, respectively. Activity of single-unit, ischemia-sensitive cardiac spinal afferents was recorded from the left sympathetic chain in anesthetized cats. Injection of 1.5 ml PRP + collagen (activated platelets) into the left atrium (LA) stimulated 12 of 13 cardiac afferents. PRP + saline (nonactivated platelets, LA) and PPP + collagen did not alter activity of these afferents. PRP + thrombin (1.5 ml, LA) stimulated eight of nine other cardiac afferents, whereas PPP + thrombin did not stimulate any of the nine afferents. Antiplatelet immune serum (1 ml/kg iv) significantly decreased circulating platelets as well as neutrophils (polymorphonuclear leukocytes, PMNs) in eight other cats, and in each animal, attenuated the ischemia-related increase in activity of cardiac afferents. Conver-
sely, responses of five separate cardiac afferents to ischemia were not diminished after treatment with anti-PMN immune serum when concentration of circulating platelets was maintained by infusion of donated PRP despite the decrease in circulating PMNs. These data indicate activated platelets stimulate ischemia-sensitive cardiac spinal afferents and contribute to activation of these afferents during ischemia.

ANGINA PECTORIS (cardiac pain) results from myocardial ischemic episodes that excite visceral sensory nerve endings in the heart. Cardiac spinal (sympathetic) afferents, including finely myelinated (Aδ) and unmyelinated (C) fibers, generally are considered the main pathway for transmitting cardiac nociception to the central nervous system during myocardial ischemia (1, 21, 19). In this regard, bilateral removal of thestellate ganglion and excision of the first to fifth thoracic symp-
pathetic ganglia, but not the nodose ganglia (vagus nerve), abolishes or relieves cardiac pain in patients with ischemic heart disease (40). Occlusion of the cor-
onary artery also produces severe pain and pseudoadaptative reactions in animals, which can be abolished by thoracic sympathectomy but not by vagotomy (21). Cardiac spinal afferents can be classified into ischemically sensitive afferents that respond to myocardial ischemia (26, 35) and ischemically insensitive afferents whose activity is not altered during occlusion of the coronary artery (15). It is generally accepted that is-
chemically sensitive cardiac spinal afferents transmit nociceptive information to the central nervous system to elicit cardiac pain perception and initiate excitatory cardiovascular reflexes including hypertension and tachyarrhythmias (15, 21, 35, 39, 40). Previously, we have demonstrated that ischemic metabolites including lactic acid (protons), bradykinin, prostaglandins, and reactive oxygen species (ROS), among others (27, 32, 15), but not adenosine (26), contribute to activation of ischemically sensitive cardiac afferents during myocardial ischemia. However, none of the previously in-
vestigated ischemic metabolites appears to be solely responsible for activation of cardiac afferents during ischemia. We believe, therefore, that other ischemic metabolites likely account for activation or sensitiza-
tion of cardiac spinal afferents during ischemia.

Thrombocytes (platelets) circulate in the blood-
stream as small, anucleate, disk-shaped cells. Platelets are activated by myocardial ischemia as shown in sev-
eral clinical and experimental studies (7, 8, 13, 24).
First, patients with spontaneous or unstable angina, or with myocardial infarction, demonstrate increased platelet aggregatability and adhesiveness and release of platelet content (8, 13). Second, animal experiments have shown that occlusion of the coronary artery is associated with activation of platelets (7, 24). When platelets are activated, they display a similar sequence of responses including shape change, aggregation, and secretion (release of granule contents) (14, 31). Previous studies have demonstrated that platelets release two major classes of mediators: 1) arachidonic acid metabolites, such as prostaglandin, and 2) biogenic amines, including serotonin and histamine (14, 20).
Prostaglandins are capable of stimulating both cardiac sympathetic and vagal afferents (23, 32, 36). We have shown previously that endogenous serotonin and histamine stimulate ischemically sensitive abdominal visceral afferents (9, 10). Moreover, Schmelz et al. (29) reported that intradermal injection of human platelets can induce a distinct pain sensation in humans. Finally, application of activated human platelet solutions to an ex vivo skin-nerve preparation of rats excites cutaneous nociceptors (28). However, despite the above observations, the role of activated platelets in excitation of cardiac spinal afferents during myocardial ischemia has not been determined.

The general aim of this study was to determine whether activated platelets play a role in excitation of ischemically sensitive cardiac spinal afferents during ischemia. We hypothesized that 1) platelets activated by collagen and thrombin, respectively, stimulate ischemically sensitive cardiac spinal afferents; and 2) depletion of circulating platelets by a polyclonal antiplatelet antibody attenuates the increased activities of cardiac spinal afferents during myocardial ischemia. In addition, in pilot studies, we observed that the antiplatelet antibody also decreased the number of neutrophils or polymorphonuclear leukocytes (PMNs). Because PMNs have been suggested to play a role in excitation of cardiac spinal afferents (33), a specific protocol on anti-PMN antibody plus infusion of platelet-rich plasma (PRP), which could deplete PMNs and restore circulating platelets, was employed to differentiate between the role of platelets and that of PMNs in the activation of these afferents during ischemia.

METHODS

Surgical Preparation

Surgical and experimental protocols used in this study were approved by the Animal Use and Care Committee at the University of California at Irvine. The studies conformed to American Physiological Society’s “Guiding Principles in the Care and Use of Animals.” Experiments were performed on 41 fasted cats of either sex (3.1 ± 0.5 kg, means ± SD). Each animal was anesthetized with ketamine (20–30 mg/kg im; Phoenix Scientific; St. Joseph, MO) and anaesthesia was maintained with α-chloralose (40–50 mg/kg iv). Additional injections of α-chloralose (5–10 mg/kg iv) were given as necessary to maintain an adequate depth of anesthesia assessed by observing the absence of a conjunctival reflex. The animal’s trachea was intubated and respiration was maintained artificially (Harvard pump, model 661; Ealing; South Natick, MA). The cat was ventilated by air supplemented with 100% O2 through the respirator. The femoral vein and artery were cannulated for the administration of drugs and fluid and measurement of blood pressure, respectively. Another catheter (polyethylene-90) was introduced into the left atrium (LA) through the left atrial appendage for intracardiac injection of solutions. Arterial blood pressure was measured by a pressure transducer (Statham P 23 internal diameter; Gould) connected to the femoral arterial catheter. Arterial blood gases were frequently assessed by a blood gas analyzer (ABL-5, Radiometer; Copenhagen, Denmark) and maintained within physiological limits (PO2 >100 mmHg, PCO2 = 28–35 mmHg, pH 7.35–7.45) by adjusting the respirator rate or tidal volume, or by intravenously administering 2–3 ml of 1 M of NaHCO3 (8.4% wt/vol). Body temperature was monitored by a rectal thermistor and maintained at 36–38°C with a circulating-water heating pad and a heat lamp. At the end of the experiment, the animals were euthanized by the administration of saturated potassium chloride into the femoral vein under deep anesthesia that was ensured by giving an additional dose of α-chloralose (50 mg/kg).

Cardiac Spinal Afferent Recordings

Impulse activity of cardiac afferents in the left sympathetic chain and rami communicates (T2 to T5) was recorded as we have described previously (9, 26, 32). In brief, a midline sternotomy was performed and the first through seventh left ribs and the left lung were removed. The left paravertebral sympathetic chain was isolated, draped over a Plexiglas platform, and covered with warm mineral oil. Small nerve filaments were dissected gently from the chain between T2 and T5 under an operating microscope (Zeiss) and the rostral ends were placed across one pole of the recording electrode. The other pole of the recording electrode was grounded with a cotton thread to the animal. The recording electrode was attached to a high impedance probe (model HIP511, Grass Instruments; Quincy, MA), and the signal was amplified (model P511 preamplifier; Grass) and processed through an audioamplifier (AM1B audiomonitor; Grass) and an oscilloscope (model 2201, Tektronix; Beavertown, OR). The signal was recorded on a chart recorder (TA 4000B, Gould; Cleveland, OH) with an IBM-compatible Pentium computer through an analog-to-digital interface card (RC Electronics; Santa Barbara, CA) for subsequent off-line analysis. The discharge frequency of afferents was analyzed by using data acquisition and analysis software (EGAA version 3.02; RC Electronics) and a histogram was created for each afferent. Accurate counting of the impulse activity of the afferent was verified for each afferent by comparing the constructed histogram with the original neurogram.

The receptive field of each afferent was located by mechanical stimulation of the heart. This included constricting the thoracic aorta as well as gently probing the heart with a cotton swab. The location of the afferent nerve ending was confirmed further by placing a stimulating electrode directly on the surface of the myocardium to evoke the action potential of the afferent. This procedure was followed by occlusion of the appropriate coronary artery or application of bradykinin to the receptive field. Conduction velocity (CV) of each afferent fiber was calculated by dividing the conduction distance by the conduction time. Conduction time was determined by measuring the delay between the triggered artifact from electrical stimulation and the action potential of the afferent detected by the recording electrode. Conduction distance was estimated by measuring the length of a wet thread placed along the presumed afferent pathway, from the receptive field along the course of the inferior cardiac nerve through the left stellate ganglion to the recording electrode on the sympathetic chain (26, 32). C- and Aδ-fiber afferents were classified as those with CVs of <2.5 and 2.5–30 m/s, respectively. In the present study, each afferent had a single receptive field that could be located precisely.

Myocardial ischemia was induced by complete occlusion of the appropriate coronary artery supplying the regional receptive field of the cardiac afferent nerve with a thread placed around the vessel. Ischemia was confirmed by observing a regional change in the color of the myocardium,
which has been closely correlated to the production of lactic acid as indicated by a reduction in tissue pH (27). Afferents were considered to be ischemically sensitive if their discharge activity during 3–5 min of myocardial ischemia was increased at least twofold above baseline activity (26, 32).

Additionally, an occlusion cuff was placed around the descending aorta to increase aortic pressure and left ventricular pressure to determine whether the single cardiac afferent was mechano- or chemosensitive.

**Platelet Activation with Agonists**

Activation of platelets was induced and assessed by observing the aggregation of platelets (visible clumping followed by gradual clearing of the initially cloudy solution), and through microscopy with a hemacytometer (Fisher Scientific; Pittsburgh, PA) in which the clumps of platelets are observed along with a large decrease in their number in PRP (2, 31). PRP and platelet-poor plasma (PPP) were prepared as described previously (2, 11). Briefly, 10 ml of blood from a donor cat was collected into a polystyrene tube containing 3.8% sodium citrate (9.1 vol/vol) and was then centrifuged at 150 g for 10 min at 22°C (Allegra 6R centrifuge; Beckman Coulter; Fullerton, CA). The supernatant PRP was removed with plastic pipettes, placed into plastic tubes, and the remaining blood sample was centrifuged at 2,500 g for 10 min at 22°C to obtain PPP. In each case, the number of platelets in PPP was adjusted to 450–600 × 10^9 platelets/l. Platelets were immediately activated by incubating PRP (37°C, 10 min) with 2 mg/ml of collagen (Type I bovine Achilles tendon; Sigma; St. Louis, MO) and 5 U/ml of thrombin (Calbiochem; La Jolla, CA), respectively, followed by agitation for 30 s on a vortex genie mixer (Scientific Products) (38). The solution of collagen was prepared as described by Packham et al. (25). Doses of collagen and thrombin used in the present study are capable of activating human and feline platelets (11, 38). Numbers of platelets were determined in the samples of PPP as well as in activated and unactivated PRP. Supernatants from the suspensions were obtained by centrifugation of PPP and activated or unactivated PRP at 2,500 g for 10 min at 22°C, respectively; these were then stored in crushed ice or at −20°C until needed.

**Platelet Isolation and Preparation of Polyclonal Antibody Against Platelets**

Washed platelet suspensions from cats were prepared as described previously (30). All centrifugation was done at 22°C. Blood was collected into sterile plastic tubes containing acid citrate-dextrose (8:1 vol/vol). After sedimentation of red blood cells (RBC) with 6% gelatin in 0.9% NaCl, the white cell/platelet-rich supernatant was centrifuged at 200 g for 10 min. Platelet-rich supernatant was removed carefully from the white cell pellet and centrifuged at 1,500 g for 15 min to obtain a platelet pellet. The platelet pellet was washed twice in 0.9% NaCl containing 0.4% sodium citrate and centrifuged at 1,500 g for 10 min. Then, the platelet pellet was resuspended in modified Hanks’ balanced salt solution (mHBSS, JRH Biosciences; Lenexa, KS) containing 3.5% bovine serum albumin (Sigma). Platelets in suspension were counted with a hemacytometer.

Polyclonal antiplatelet antibody was produced in rabbits immunized with an initial subcutaneous injection of 10^8 platelets in Freund’s complete adjuvant (Pierce; Rockford, IL). Three additional boosters of 10^8 platelets in Freund’s incomplete adjuvant were given at 2-wk intervals. Two weeks after completion of the immunization series, blood was collected from rabbits and the antiserum was obtained. As previously described by Drenckhahn et al. (3), the polyclonal antiplatelet antibody (immunoglobulin) was purified from rabbit serum using saturated ammonium sulfate precipitation. In addition, normal serum collected from nonimmunized rabbits was used as a control. The antiplatelet antibody and serum were stored at −70°C until use.

**Neutrophil Isolation and Preparation of Polyclonal Antibody Against PMNs**

Washed PMNs were isolated from whole blood of cats using a modified Percoll density gradient method as described by Weyrich et al. (39) and Tjen-A-Looi et al. (33). Briefly, venous blood was collected into plastic tubes containing acid citrate-dextrose (8:1 vol/vol), then was mixed (1:1) with Dulbecco’s PBS (Sigma). After an addition of 6% medical grade dextran (1:5, Sigma), RBCs were allowed to settle. The upper plasma fraction (leukocyte cells/PRP) was pipetted from the RBCs and was then centrifuged at 400 g for 20 min. Supernatant (PRP) was decanted from the leukocyte pellet and was centrifuged at 2,500 g for 10 min to obtain PPP. The leucocyte pellet was washed with modified mHBSS (JRH Biosciences) and centrifuged at 400 g for 10 min. Contaminating RBCs were depleted from the leucocyte pellet with hypotonic lysis and the suspension was recentrifuged. Isotonic percoll (Sigma) was made by mixing Percoll with 1.5 M NaCl (9:1). The isotonic Percoll was mixed with PPP to produce three solutions with 80, 62, or 50% Percoll. Starting with the most dense solution of Percoll, 4 ml of each solution was layered in a 15-ml polypropylene conical tube. Leukocytes were resuspended in PBS, then layered on the top of the gradient and centrifuged at 1,500 g for 40 min. PMNs were collected at the 62–80% interface and washed with PBS. The PMN pellet was resuspended in PBS and was checked for the predominance of PMNs with hematoxylin and eosin stain. Finally, the PMNs were mixed with either Freund’s complete or incomplete adjuvant (1:1; Pierce) for producing the polyclonal anti-PMN antibody.

Polyclonal anti-PMN antibodies were produced in rabbits immunized with the purified PMNs injected subcutaneously initially and 3–4 wk later using Freund’s complete (initial immunization) and incomplete (followup immunization) adjuvant. Two weeks after completion of the immunization series, blood was collected from the rabbits and antiserum was obtained. As previously described by Drenckhahn et al. (3), the polyclonal anti-PMN antibody (immunoglobulin) was purified from rabbit antiserum using saturated ammonium sulfate precipitation. The anti-PMN antibody was stored at −70°C until use.

**Experimental Protocols**

**Platelet activation by collagen and thrombin.** PRP and PPP were collected from 22 cats, including 13 for PRP + collagen, and 9 for PRP + thrombin. PRP was activated by collagen (PRP + collagen, 2 mg/ml) and thrombin (PRP + thrombin, 5 U/ml), respectively. We verified activation of platelets by observing the formation of platelet clumps and a large decrease in the number of platelets (from 450–600 × 10^9 to <60–80 × 10^9 platelets/l) in PRP under microscopy with the hemacytometer (Fisher). Supernatants from activated PRP, unactivated PRP, and PPP solutions were used in the cardiac afferent study.

**Responses of ischemically sensitive cardiac afferents to activated platelets.** This protocol examined the effect of activated platelets on the discharge frequency of ischemically
sensitive cardiac afferents in two groups of cats. In the first group of 13 animals, after the location of the receptive field of an afferent fiber was established in the ventricles, the response of the cardiac afferent was identified during 5 min of ischemia induced by occlusion of the regional coronary artery. A minimum 30-min recovery period was used to allow baseline activity of the afferent to be reestablished. Subsequently, we examined the responses of each afferent to 1.5 ml of PRP + collagen (2 mg/ml, activated platelets), 1.5 ml of PPP + collagen (2 mg/ml) and 1.5 ml of PRP + saline (nonactivated platelets). Each solution was administered into the LA 20 min apart in random order.

The second group of nine afferents was studied in an identical fashion to the first group with the exception that platelets were activated by thrombin (5 U/ml) in place of collagen.

Effect of depletion of circulating platelets on responses of cardiac afferents to myocardial ischemia. In this protocol, cardiac afferents were subjected to two 5-min periods of myocardial ischemia separated by 5-min of reperfusion and 35–45 min of recovery. These intervals are capable of inducing reproducible responses from cardiac afferents without damaging or sensitizing nerve endings (32).

ANTIPATELET ANTIBODY. The polyclonal antiplatelet antibody (1 ml/kg) was diluted by 20 ml of 0.9% NaCl and was then infused into the femoral vein at 1 ml/min for 20–25 min during the recovery period after the first period of ischemia. Circulating platelets and PMNs in whole blood were measured in complete blood counts using the QBC centrifugal hematology system (Becton Dickinson; Franklin Lakes, NJ). In a pilot study, we observed that this dose of antiplatelet antibody effectively depletes circulating platelets (>92%) in cats within 45 min. To a smaller degree, circulating PMNs were also reduced (51%) by this antibody. After confirming the reduction in circulating platelets in eight cats, we repeated 5 min of myocardial ischemia followed by reperfusion. Responses of afferents during infusion of the antiplatelet antibody and to repeated ischemia were recorded.

TIME CONTROL. A second group of six animals was tested identically to the antiplatelet antibody group with the exception that normal rabbit serum (1 ml/kg) was administrated during the first recovery period. Blood cell counts also were measured in this group.

PRP + POLYCLONAL ANTI-PMN ANTIBODY. We initially, but unsuccessfully, attempted to restore the concentration of platelets in animals tested with the antiplatelet antibody in three cats. Second, we attempted to restore the concentration of PMNs by perfusion of neutrophil-rich plasma in two cats with the antiplatelet antibody, but we again failed. Therefore, we developed a polyclonal anti-PMN antibody that depleted circulating PMNs and reduced platelets to a smaller extent. We observed that the administration of the anti-PMN antibody in conjunction with infusion of PRP allowed us to maintain the concentration of circulating platelets. Thus the anti-PMN antibody (1 ml/kg diluted by 20 ml of saline) was infused intravenously during the recovery period after the first period of ischemia. Then 20–30 ml of PRP from donor cats were infused intravenously. Blood cell counts were measured to confirm the reduction of PMNs to <8% of control levels while maintaining platelets to >82% of control levels. This level of reduction in circulating neutrophils with the anti-PMNs antibody is significantly greater than that observed with the administration of the antiplatelet antibody. Subsequently, myocardial ischemia was repeated for 5 min. Responses of the afferents during the administration of the anti-PMN antibody plus PRP and during repeated ischemia were recorded.

Data Analysis

Discharge rates of cardiac spinal afferents were expressed in impulses/s and were averaged during 5 min of preischemia, 5 min of ischemia, and the initial 2 min of reperfusion (26, 32). We measured the response of the afferents to PRP + collagen, PRP + thrombin, PRP + saline, PPP + collagen, and PPP + thrombin by averaging discharge rates of the afferents during the entire period of response defined as the time during which sustained activity exceeded baseline activity by 20%. We assessed the latency of afferent response to each stimulus from the time of LA injection of the solutions to the point when sustained discharge activity of afferents exceeded baseline activity by 20%. Baseline activity was determined over a 5-min period of preischemia.

Data are expressed as means ± SE. The effect of collagen and thrombin on the number of platelets in PRP, and the effects of PRP + collagen, PPP + collagen, PRP + saline, PRP + thrombin, and PPP + thrombin on the activity of cardiac afferents were compared using one-way repeated-measures ANOVA with Tukey’s post hoc test. The same tests were used to examine the influence of the antiplatelet antibody, rabbit serum, and anti-PMN antibody plus PRP on circulating platelets and PMNs and on ischemia-induced responses of the afferents. The effects of the anti-PMN antibody on circulating platelets and PMNs also were compared with the effects of antiplatelet antibody using one-way ANOVA with Tukey’s post hoc test. If data were not normally distributed, as determined by the Kolmogorov-Smirnov test, they were compared with the Friedman ANOVA on ranks with Dunnett’s post hoc test. All statistical calculations were performed with SigmaStat software (Jandel Scientific Software; San Rafael, CA). Values were considered to be significantly different when P < 0.05.

RESULTS

Responses of Cardiac Afferents to Activated Platelets

We observed formation of clumps of platelets and a significant reduction of platelets in the samples of PRP treated with collagen (Fig. 1A) or thrombin (Fig. 1B). In contrast, in the samples of PRP + saline there were no such changes in the concentration of platelets. These observation provided evidence of platelet activation (31).

Figure 2 displays representative tracings of an ischemically sensitive afferent with a CV of 1.32 m/s inner-vating the posterior wall of the left ventricle. Injection of 1.5 ml of PRP + collagen, but not PPP + collagen into the LA, increased discharge activity of this afferent from 0.43 to 1.78 impulses/s after an onset latency of 15 sec.

Five minutes of myocardial ischemia increased the discharge frequency of 13 cardiac afferents (4 Aδ fibers and 9 C fibers) from 0.28 ± 0.04 to 1.68 ± 0.26 impulses/s. Injection of 1.5 ml of PRP + collagen into the LA stimulated 12 of the 13 cardiac afferents (4 Aδ fibers, CV = 5.02 ± 0.50 m/s; 9 C fibers, CV = 1.09 ± 0.18 m/s) significantly increasing their discharge activity from 0.39 ± 0.06 to 1.35 ± 0.12 impulses/s after an onset latency of 10 ± 1.0 s (Fig. 1C). In contrast,
injection of PRP + saline or PPP + collagen into the LA did not stimulate any of the 13 afferents studied (0.41 ± 0.07 vs. 0.40 ± 0.07 and 0.44 ± 0.07 vs. 0.45 ± 0.09 impulses/s before vs. after, respectively). Most (92%) of these afferents did not respond to an increase in arterial pressure (93 ± 13 to 169 ± 16 mmHg) induced by partial occlusion of descending aorta. Location of the receptive fields of each of the 13 afferent nerve endings is shown in Fig. 7.

Figure 1D summarizes the effect of activated platelets with thrombin on ischemically sensitive cardiac spinal afferents in nine cats. Injection of 1.5 ml of PRP + thrombin into the LA stimulated eight of nine cardiac afferents (2 Aβ fibers, CV = 5.92 ± 0.03 m/s; 7 C fibers, CV = 1.21 ± 0.24 m/s) significantly increasing their discharge activity from 0.44 ± 0.10 to 1.28 ± 0.18 impulses/s after an onset latency of 9.6 ± 1.4 sec. Conversely, injection of PRP + saline and PPP + thrombin into the LA did not stimulate any of the nine afferents (0.38 ± 0.09 to 0.37 ± 0.07, and 0.37 ± 0.08 vs. 0.42 ± 0.09 impulses/s, before vs. after, respectively). Eight of the nine afferents did not respond to arterial pressure increase (91 ± 12 to 171 ± 14 mmHg) induced by partial occlusion of descending aorta. Each of the afferent nerve endings were located in the left ventricle (Fig. 7).
Depletion of Circulating Platelets on the Response of Cardiac Afferents to Myocardial Ischemia

Figure 3 summarizes the effect of rabbit serum and the antiplatelet antibody on circulating platelets and PMNs in cats. Concentration of platelets was not altered in cats that received rabbit serum (Fig. 3A). Although brief myocardial ischemia (initial and repeat) did not significantly change the circulating platelets, the number of platelets was decreased dramatically from $315 \pm 20$ to $21 \pm 4 \times 10^9$ platelets/l after infusion of antiplatelet antibody (Fig. 3B). We also observed that the concentration of PMNs was decreased significantly from $14 \pm 1.2$ to $6 \pm 0.4 \times 10^9$ PMNs/l after infusion of the antiplatelet antibody (Fig. 3B), whereas the concentration of PMNs was not changed in cats that received rabbit serum (Fig. 3A).

A representative histogram displaying the response of a cardiac spinal afferent ($CV = 0.62$ m/s) innervating the posterior wall of the left ventricle to 5 min of myocardial ischemia before and after treatment with the antiplatelet antibody is shown in Fig. 4. Myocardial ischemia increased baseline activity of this afferent from 0.20 to 1.98 impulses/s (Fig. 4A). Treatment with the antiplatelet antibody (1 mg/kg iv) significantly decreased the numbers of circulating platelets from $385 \times 10^9$ to $5 \times 10^9$ platelets/l and attenuated the increase in discharge activity of this afferent compared with the initial period of ischemia (Fig. 4B). Also, the numbers of circulating PMNs were reduced from $15 \times 10^9$ to $7.6 \times 10^9$ PMNs/l by the administration of antiplatelet antibody.

Figure 5 summarizes the influence of treatment with rabbit serum and the antiplatelet antibody on the impulse activity of 14 ischemically sensitive cardiac
afferents (4 Aβ fibers, CV = 4.88 ± 0.58 m/s; 10 C fibers, CV = 1.03 ± 0.16 m/s) during the entire 5 min of ischemia. Before treatment, ischemia significantly increased the discharge activity of these afferents from 0.17 ± 0.06 to 1.66 ± 0.13 impulses/s. Depletion of circulating platelets by the antiplatelet antibody significantly attenuated the response to ischemia in the eight responsive cardiac afferents (Fig. 5B). Similar to the change in the mean discharge activity, summed activity of all afferents during the entire 5 min of ischemia was attenuated by 32.4% after the administration of the antiplatelet antibody compared with the period before treatment with the antibody (Fig. 5, Cb and Ca). This attenuation in the response of afferents to a second period of ischemia likely was not the result of a general decrease in reactivity over time, because another group of six cardiac afferents (1 Aδ fibers, CV = 3.9 m/s; 5 C fibers, CV = 0.95 ± 0.20 m/s) were consistently responsive to 5 min of ischemia (0.19 ± 0.04 to 1.61 ± 0.24 vs. 0.24 ± 0.05 to 1.72 ± 0.26 impulses/s, initial vs. repeat ischemia after infusion of normal rabbit serum) (Fig. 5A). Additionally, cardiac afferents did not respond to the administration of rabbit serum in the absence of ischemia. The location of each receptive field of the afferent nerve endings studied in this protocol is shown in Fig. 7.

Effect of PRP Infusion Plus Anti-PMN Antibody on Circulating Platelets and Response of Cardiac Afferents to Myocardial Ischemia

Figure 6 summarizes the effect of infusion of PRP plus the anti-PMN antibody on the concentration of both circulating platelets and PMNs and on the impulse activity of five cardiac afferents (1 Aδ fibers CV = 3.83 m/s; 4 C fibers, CV = 0.76 ± 0.21 m/s) during ischemia. In the absence of anti-PMNs antibody, ischemia significantly increased the discharge activity of platelets and polymorphonuclear leukocytes (PMNs) to the PMN antibody (1 ml/kg iv, n = 8) on mean (5 min) discharge frequency of 8 cardiac afferents before and during 5 min of ischemia. C: histograms show summated 5-s impulse activity of the above 8 afferents during 5 min of ischemia before (a) and after (b) treatment with antiplatelet antibody. Data are presented as means ± SE. *P < 0.05, vs. preischemia; †P < 0.05, pre- vs. postantiplatelet antibody.

Fig. 5. A: effect of repeat ischemia on mean (5 min) impulse activity of 6 cardiac spinal afferents before and during 5 min of myocardial ischemia in the absence and the presence of normal rabbit serum. B: bar graph summarizes the effect of antiplatelet antibody (1 ml/kg iv, n = 8) on mean (5 min) discharge frequency of 8 cardiac afferents before and during 5 min of ischemia. C: histograms show summated 5-s impulse activity of the above 8 afferents during 5 min of ischemia before (a) and after (b) treatment with antiplatelet antibody. Data are presented as means ± SE. *P < 0.05, vs. preischemia; †P < 0.05, pre- vs. postantiplatelet antibody.
these afferents from $0.19 \pm 0.06$ to $1.53 \pm 0.12$ impulses/s (Fig. 6B). The afferent nerve endings were located in the left ventricle as displayed in Fig. 7. In the presence of the anti-PMNs antibody, infusion of PRP maintained circulating platelets at $256 \pm 19 \times 10^9$ platelets/l, which was close to the control level (i.e., pre-anti-PMN antibody, $286 \pm 20 \times 10^9$ platelets/l) and did not raise the concentration of circulating PMNs (Fig. 6A). After treatment with this antibody plus PRP, the response to ischemia in the five responsive cardiac afferents (Fig. 6B) was not changed significantly compared with the initial ischemia ($1.53 \pm 0.12$ vs. $1.84 \pm 0.25$ impulses/s, pre-anti-PMNs antibody + PRP vs. post-anti-PMNs antibody PRP). Also, the activity of these spinal afferents was unchanged during either the administration of the anti-PMN antibody or infusion of PRP.

**DISCUSSION**

Two main observations were made in the present study. First, activated platelets are capable of stimulating ischemically sensitive cardiac spinal afferents. In this regard, we observed that injection of platelets activated either by collagen or thrombin into the LA stimulated ischemically sensitive cardiac spinal afferents. Second, increases in the responses of cardiac afferents during ischemia were attenuated significantly when the concentration of circulating platelets was decreased to minimal levels in cats after the administration of an antiplatelet antibody. This is the first study to demonstrate that activated platelets contribute to the excitation of cardiac spinal afferents during brief myocardial ischemia.

Myocardial ischemia is associated with activation of cardiac spinal afferents. However, cell types that contribute to these responses have not yet been identified. Previous studies have shown that protons, bradykinin, and ROS, but not adenosine, contribute to activation of ischemically sensitive spinal afferents (15, 26, 27, 32). We believe, however, that still other mechanisms account for activation/sensitization of cardiac afferents during ischemia, because inhibition of these stimuli does not fully eliminate the responses of these afferents to ischemia.

Platelet activation occurs during myocardial ischemia in experimental animals with coronary artery occlusion (7, 24). Patients with spontaneous or unstable angina, as well as those experiencing myocardial infarction, also manifest platelet activation (6, 8, 13). Four well-defined stages of activation of platelets are recognized: 1) adhesion, 2) shape change, 3) contraction and release of granule contents, and 4) aggregation (14, 31). Although circulating freely in the blood under conditions of normal homeostasis, platelets interact with cell matrix components like collagen, fibronectins, laminin, and von Willebrand factor, as well as with thrombin, ADP, and epinephrine when vascular injury occurs (31). These interactions stimulate specific receptors and activate the glycoprotein IIb/IIIa (αIIbβ3) integrins on the plasma membrane of platelets, thereby leading to platelet activation (31, 34). During myocardial ischemia and infarction, rupture of the atherosclerotic plaque exposes components of the arterial media (particularly fibrillar collagen) to blood components resulting in activation of both platelets and the coagulation system. Stimulation of the clotting system generates thrombin, which further promotes platelet aggregation (8, 13).

Platelets contain several mediators in their dense granules, α-granules, and lysosomal vesicles. Dense granules contain mostly small molecules and ions including ATP and ADP, 5-hydroxytryptamine (5-HT), calcium, inorganic diphosphate, and inorganic phosphate (14, 22). Lysosomal vesicles and α-granules primarily contain macromolecular substances including proteins, glycoproteins, proteoglycans, and a number of different acid hydrolytic enzymes (14, 31). The contents of these granules are released when platelets are activated by stimulants including ischemia, agonists, and contact with various natural and artificial surfaces. Weak agonists such as ADP, epinephrine, 5-HT, and vasopressin cause release of granule contents, a process dependent on the formation of arachidonate metabolites, prostaglandin endoperoxides (PGG2/PGH2), or thromboxane A2 (14, 31). Strong agonists such as collagen, thrombin, the calcium ionophore A-23187, and phorbol esters can induce secretion of granules, a procedure independent of arachidonic acid metabolites (14, 31, 37). Concentration of free 5-HT in human whole blood increases from 36 to 836 nM after activation of platelets with collagen (16). Adding thrombin to PRP stimulates platelets to release 5-HT (2). In addition, the concentration of 5-HT is elevated in coronary sinus blood of patients with angina and after brief coronary occlusion with angioplasty (12, 37). We have shown that 5-HT stimulates ischemically sensitive abdominal spinal afferents (9). Cyclooxygenase products, particularly PGE2 and possibly PGI2, likewise have the capability of stimulating both cardiac sympathetic and vagal afferents (23, 32, 35). Therefore, there was a
substantial rationale for hypothesizing that activation of platelets associated with ischemia contributes to excitation of cardiac spinal afferents during myocardial ischemia.

To assess the potential for activated platelets to stimulate ischemically sensitive cardiac spinal afferents, we examined the effects of platelets activated by either collagen or thrombin on the activity of cardiac afferents. Collagen and thrombin are recognized agonists that activate platelets through separate second messenger mechanisms (14, 31). Thrombin activates platelets without a measurable increase in cytoplasmic Ca$^{2+}$ of platelets, whereas collagen activates platelets by increasing cytoplasmic Ca$^{2+}$ (14). In the present study, we found that the administration of platelets activated by collagen or thrombin into the LA increased the discharge activity of ischemically sensitive cardiac spinal afferents. This finding was consistent with that of Ringkamp et al. (28) who demonstrated that application of platelets activated by ADP to the receptive field of cutaneous afferents in rats enhanced the discharge activity of the somatic C-fiber afferents. Others have documented that intravenous injection of a platelet-rich solution induces a graded burning pain and protracted hyperalgesia in humans (29). Conversely, activated platelets decrease the sensitivity of baroreceptors in the carotid sinus of rabbits (18). Thus the influence of activated platelets on sensory nerves has led to mixed results.

An important goal in this study was to determine whether activation of platelets during myocardial ischemia contributes to excitation of cardiac spinal afferents. Previously, Leanos et al. (17) demonstrated that depletion of circulating platelets by antiplatelet antibody abolished the reflex pulmonary pressor and systemic depressor responses to intravenous injection of autologous bone marrow. In the present study, we developed a rabbit polyclonal antiplatelet antibody capable of substantially reducing the concentration of circulating platelets from control level 315 ± 20 × 10$^3$ platelets/l to <8% of control level in cats. Reduction of circulating platelets substantially attenuated the responses of cardiac spinal afferents to brief myocardial ischemia. Conversely, control rabbit serum did not alter the response of cardiac spinal afferents to brief ischemia. Golino et al. (12) observed that brief myocardial ischemia induced by coronary angioplasty leads to activation of platelets. Thus platelets activated during myocardial ischemia contribute to excitation of cardiac spinal afferents during ischemia.

A confounding observation arising from our study relates to the reduction of both circulating platelets and PMNs after the administration of the polyclonal antiplatelet antibody. Decrease in circulating PMNs could contribute to the attenuated cardiac afferent responses during ischemia, because PMNs may be the sources of ROS in the heart. PMNs generate ROS as a host defense mechanism and are attracted to the heart during ischemia and reperfusion (4, 5). Previous studies from our laboratory and others have shown that ROS, including -OH, H$_2$O$_2$, and superoxide radical, activate both sympathetic and vagal sensory systems in the abdominal and cardiac regions in the absence of injury (15, 36). To differentiate between the role of activated platelets and that of PMNs, we tried several approaches. First, we tried to restore the circulating platelets to the control level by infusion of donated PRP after antiplatelet antibody. However, we were unable to restore the platelet concentration by perfusion of PRP within a 6-h time frame after the administration of antiplatelet antibody in three cats. Also, we unsuccessfully attempted to restore the neutrophil concentration by infusing white blood cell-rich plasma in two other cats treated by the antiplatelet antibody. We then developed a polyclonal anti-PMN antibody that reduced circulating PMNs to a greater extent than observed with the antiplatelet antibody. Infusion of PRP after the administration of the polyclonal anti-PMN antibody allowed maintenance of the concentration of circulating platelets. We observed that the responses of cardiac spinal afferents to repeat ischemia after the anti-PMNs antibody plus PRP was not altered compared with the initial ischemia before treatment with the anti-PMN antibody. These data are consistent with our recent study showing that PMNs are not an important mechanism in the ROS-mediated activation of cardiac spinal afferents during ischemia (33). Therefore, we believe that attenuation of the responses of cardiac spinal afferents to myocardial ischemia after treatment with the antiplatelet antibody is the result of the depletion of circulating platelets, but not PMNs.

In conclusion, the present study has demonstrated that platelets activated by either collagen or thrombin are capable of stimulating ischemically sensitive cardiac spinal afferents. More importantly, depletion of circulating platelets attenuates the response of cardiac spinal afferents to myocardial ischemia. These data suggest that activation of platelets during myocardial ischemia is responsible in part for excitation of cardiac spinal afferents. Increased activity of this nerve system is associated with cardiac pain and excitatory reflex cardiovascular responses including hypertension and cardiac tachyarrhythmias that accompany myocardial ischemia. The influence of mediators released by activated platelets on stimulation of cardiac spinal afferents will require further investigation.

We gratefully acknowledge the technical assistance of Melissa Crisostomo, Ngoc Phan, and Liying Zhang. We thank Mark Causin, a student fellow of the American Heart Association, Western States Affiliate, for active participation in this project. We also thank Nicholas Nelson for editorial assistance.

This study was supported by National Heart, Lung, and Blood Institute Grants HL-36527 and HL-51428 and by Beginning Grant-in-Aid 9960007Y from the American Heart Association, Western States Affiliate.

Part of this work was presented as a preliminary communication at the 72nd American Heart Association Scientific Sessions, Atlanta, GA, 1999.

REFERENCES


