

# Xanthine oxidase, but not neutrophils, contributes to activation of cardiac sympathetic afferents during myocardial ischaemia in cats

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Activation of cardiac sympathetic afferents during myocardial ischaemia causes angina and induces important cardiovascular reflex responses. Reactive oxygen species (ROS) are important chemical stimuli of cardiac afferents during and after ischaemia. Iron-catalysed Fenton chemistry constitutes one mechanism of production of hydroxyl radicals. Another potential source of these species is xanthine oxidase-catalysed oxidation of purines. Polymorphonuclear leukocytes (PMNs) also contribute to the production of ROS in some conditions. The present study tested the hypothesis that both xanthine oxidase-catalysed oxidation of purines and neutrophils provide a source of ROS sufficient to activate cardiac afferents during ischaemia. We recorded single-unit activity of cardiac afferents innervating the ventricles recorded from the left thoracic sympathetic chain (T1–5) of anaesthetized cats to identify the afferents' responses to ischaemia. The role of xanthine oxidase in activation of these afferents was determined by infusion of oxypurinol ( $10 \text{ mg kg}^{-1}$ , i.v.), an inhibitor of xanthine oxidase. The importance of neutrophils as a potential source of ROS in the activation of cardiac afferents during ischaemia was assessed by the infusion of a polyclonal antibody ( $3 \text{ mg ml}^{-1} \text{ kg}^{-1}$ , i.v.) raised in rabbits immunized with cat PMNs. This antibody decreased the number of circulating PMNs and, to a smaller extent, platelets. Since previous data suggest that platelets release serotonin (5-HT), which activates cardiac afferents through a serotonin receptor (subtype 3,5-HT<sub>3</sub> receptor) mechanism, before treatment with the antibody in another group, we blocked 5-HT<sub>3</sub> receptors on sensory nerve endings with tropisetron ( $300 \text{ } \mu\text{g kg}^{-1}$ , i.v.). We observed that oxypurinol significantly decreased the activity of cardiac afferents during myocardial ischaemia from  $1.5 \pm 0.4$  to  $0.8 \pm 0.4$  impulses  $\text{s}^{-1}$ . Similarly, the polyclonal antibody significantly reduced the discharge frequency of ischaemically sensitive cardiac afferents from  $2.5 \pm 0.7$  to  $1.1 \pm 0.4$  impulses  $\text{s}^{-1}$ . However, pre-blockade of 5-HT<sub>3</sub> receptors eliminated the influence of the antibody on discharge activity of the afferents during ischaemia. This study demonstrates that ROS generated from the oxidation of purines contribute to the stimulation of ischaemically sensitive cardiac sympathetic afferents, whereas PMNs do not play a major role in this process.

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Myocardial ischaemia and reperfusion are associated with cardiovascular reflex responses as well as with chest pain. During ischaemia, activation of cardiac vagal afferents elicits reflex inhibitory cardiovascular reflexes consisting of decreases in arterial blood pressure, heart rate, and systemic vascular resistance (Oberg & Thoren, 1973). In contrast, activation of cardiac sympathetic (spinal) afferents evokes reflex excitatory cardiovascular responses (Peterson & Brown, 1971; Malliani *et al.* 1972; Huang *et al.* 1995b; Tjen-A-Looi *et al.* 1998; Fu & Longhurst, 2001). Clinical evidence suggests that angina pectoris can be relieved by stellate ganglionectomy or dorsal rhizotomy, but not by cervical vagotomy, indicating that cardiac nociception is transmitted by cardiac sympathetic afferents through spinal cord pathways (Birkitt *et al.* 1965; Palumbo &

Lulu, 1965; Meller & Gebhart, 1992). Thus, dual neural innervation of sympathetic and vagal afferents relays information from the heart to the brain.

Myocardial ischaemia and reperfusion produce a number of metabolites, including lactic acid, bradykinin (BK), prostaglandins, adenosine, and reactive oxygen species (ROS), that can stimulate cardiac afferent nerve endings (Kimura *et al.* 1977; Berger *et al.* 1977; Hirsh *et al.* 1981; Meller & Gebhart, 1992; Grill *et al.* 1992). Exogenous application of these endogenous substances sensitizes and/or activates vagal and cardiac sympathetic afferents (Brown, 1967; Staszewska-Barczak *et al.* 1976; Baker *et al.* 1980; Pagani *et al.* 1985; Pal *et al.* 1989; Nganele & Hintze, 1990). For instance, we have shown that ischaemically sensitive cardiac sympathetic afferents are activated

by endogenously produced BK (Huang *et al.* 1995a; Tjen-A-Looi *et al.* 1998), through the kinin B<sub>2</sub>-receptor (Tjen-A-Looi *et al.* 1998). Studies from other laboratories suggest that cyclooxygenase products enhance BK-induced cardiac–cardiovascular reflexes (Staszewska-Barczak *et al.* 1976). However, BK does not fully depend on prostaglandins to activate cardiac sympathetic afferents during myocardial ischaemia (Tjen-A-Looi *et al.* 1998). In contrast to BK, adenosine produced during myocardial ischaemia does not activate cardiac sympathetic afferents in cats (Pan & Longhurst, 1995). Recently, we have demonstrated that ROS are produced during brief ischaemia and reperfusion in the cat heart (O'Neill *et al.* 1996) and activate ischaemically sensitive cardiac sympathetic afferents to reflexly increase heart rate, arterial blood pressure, and myocardial contractility (Huang *et al.* 1995a,b). However, the sources that contribute to the generation of ROS and hence activation of cardiac sympathetic afferents during myocardial ischaemia are uncertain.

Ischaemia and reperfusion of the heart lead to the generation of several ROS, including hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide radicals (O<sub>2</sub>•<sup>-</sup>; Grill *et al.* 1987), and hydroxyl radicals (•OH); the latter species is formed by the Haber-Weiss reaction in the presence of iron (Halliwell & Gutteridge, 1990). Huang *et al.* (1995a) have shown that dimethyl thiourea (a chelator of several ROS species, including O<sub>2</sub>•<sup>-</sup>, H<sub>2</sub>O<sub>2</sub>, •OH and HOCl) and deferoxamine (an iron chelator and a O<sub>2</sub>•<sup>-</sup> and H<sub>2</sub>O<sub>2</sub> scavenger) attenuate the responses of ischaemically sensitive cardiac sympathetic afferents. By contrast, iron-loaded deferoxamine, which scavenges O<sub>2</sub>•<sup>-</sup> and H<sub>2</sub>O<sub>2</sub>, does not attenuate the responses of these afferents during myocardial ischaemia. These data suggest that a number of ROS may play a role, although •OH appears to be particularly important in activating cardiac afferents during ischaemia and reperfusion.

Even though ROS scavengers reduce the responses of cardiac afferents during ischaemia, the mechanisms principally involved in the production of ROS are largely unknown. In addition to the Haber-Weiss reaction, purine metabolism is one potential source of ROS. During ischaemia the purine metabolites, hypoxanthine and xanthine, accumulate from the breakdown of ATP (Jennings *et al.* 1981). Xanthine oxidase converts hypoxanthine to xanthine and can be inhibited by oxypurinol. Oxypurinol thus can decrease the synthesis of ROS like O<sub>2</sub>•<sup>-</sup> and •OH during asphyxia/reventilation and anoxia/reoxygenation, respectively (Pourcyrus *et al.* 1993; Zweier *et al.* 1994). We therefore hypothesized that the inhibition of xanthine oxidase would reduce the activity of cardiac sympathetic afferents during myocardial ischaemia.

Neutrophils (polymorphonuclear leukocytes (PMNs)) constitute another potential source of ROS during myocardial ischaemia. Mounting evidence indicates that

PMNs mediate irreversible injury of myocytes after prolonged myocardial ischaemia (Mullane *et al.* 1985; Romson *et al.* 1983). PMNs contain membrane-bound reduced forms of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that produce O<sub>2</sub>•<sup>-</sup>, which has antimicrobial action (Ferrari, 1994). O<sub>2</sub>•<sup>-</sup> released by activated PMNs amplifies the inflammatory response by the activation of a chemotactic factor, which allows activated PMNs to attach to endothelium, leading to injury of tissue by releasing additional oxidative enzymes such as myeloperoxidase and hydrolytic enzymes like elastase (Ferrari, 1994). Inhibition of PMN activity limits myocardial infarct size in pigs (Amsterdam *et al.* 1993). If PMNs produce sufficient ROS to induce tissue injury, they also could supply sufficient ROS to stimulate cardiac afferent endings during ischaemia and reperfusion. Therefore, we investigated the hypothesis that PMNs constitute an important source of ROS that stimulate cardiac sympathetic afferents during ischaemia.

To determine the role of PMNs in the activation of cardiac afferents, we used a polyclonal antibody to decrease the number of circulating PMNs. Our preliminary data indicated that the antiserum directed against PMNs to a smaller extent also decreased the number of circulating platelets. Since other data from our laboratory suggest that platelets likely play a role in the activation of cardiac sympathetic afferents during myocardial ischaemia through a serotonin subtype 3 receptor (5-HT<sub>3</sub>) mechanism (Fu & Longhurst, 2000), we performed an additional experiment to eliminate the influence of platelets; this procedure allowed us to determine if, in the absence of a 5-HT-related influence from platelets, PMNs play a major role in the activation of cardiac sympathetic afferents. These data have been presented in preliminary form (Longhurst & Tjen-A-Looi, 1998).

## METHODS

### Surgical preparation

All experiments were performed in cats of either sex (2.3–5.9 kg). Surgical and experimental protocols used in this study were approved by the Animal Use and Care Committees at the University of California, Davis and Irvine. Anaesthesia was induced with ketamine (20–30 mg kg<sup>-1</sup>, i.m.) and maintained with  $\alpha$ -chloralose (40–60 mg kg<sup>-1</sup>, i.v.). The trachea was intubated and respiration was maintained artificially (model 661, Harvard Apparatus, Ealing, South Natick, MA, USA). A femoral artery and vein were cannulated for measurement of blood pressure and administration of fluids and drugs, respectively. Arterial blood pressure was measured with a pressure transducer (Statham P23 ID, Gould, Cleveland, OH, USA) connected to the arterial catheter.

At the end of the experiments, animals were killed with saturated KCl injected into the circulation under deep anaesthesia. Deep anaesthesia was assured by giving an additional dose of  $\alpha$ -chloralose (50 mg kg<sup>-1</sup>) 5 min before administering the KCl solution.

A midline sternotomy was performed, and the first to seventh left ribs and the left lung were removed. An occlusion cuff was placed around the descending thoracic aorta. Fascia overlying the left paravertebral sympathetic chain from T2 to T6 was removed. The sympathetic chain was isolated, draped over a mirror platform and covered with warm mineral oil. Small nerve filaments were teased gently from the chain between T2 and T5 with the use of an operating microscope (Zeiss, Germany) and the rostral ends were placed individually across a recording electrode. One pole of the recording electrode was grounded with a cotton thread to the animal. The recording electrode was attached to a high-impedance probe (Grass Instruments, Quincy, MA, USA). The signal was amplified (model P511 preamplifier, Grass Instruments) and processed through an audio amplifier (model AM8B audio monitor, Grass Instruments) and fed into an oscilloscope (model 549, Tektronix, Beavertown, OR, USA) for observation. The signal was recorded on a physiograph (model TA 4000, Gould, Valley View, OH, USA) and analysed offline with a computer using an EGAA program (RC Electronics, Santa Barbara, CA, USA). The location and conduction latency of each afferent nerve ending was confirmed by electrical stimulation through an electrode placed on the afferent's receptive field in the wall of the ventricle (Pan & Longhurst, 1995). Conduction distance was estimated 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. C- and A $\delta$ -fibre afferents were classified as those with conduction velocities of < 2.5 and 2.5–30 m s<sup>-1</sup>, respectively (Huang *et al.* 1995a; Pan & Longhurst, 1995).

Myocardial ischaemia was induced by constricting the coronary artery supplying the receptive field of cardiac ventricular afferents with a thread placed around the vessel (Huang *et al.* 1995a; Pan & Longhurst, 1995; Tjen-A-Looi *et al.* 1998). Regional decreases in blood flow were confirmed by observing a regional change in colour of the myocardium. Our laboratory has shown previously that this observation is associated with lactic acid production as denoted by a local reduction in tissue pH (Pan *et al.* 1999). Afferents were considered to be ischaemically sensitive if their discharge frequency during 5 min of myocardial ischaemia was increased and sustained at least twofold above baseline activity. All sensory endings of ischaemically sensitive afferents included in this study were located either in the left or right ventricle.

Arterial blood gases were analysed every hour during the search for cardiac afferents as well as before and after each period of myocardial ischaemia with a Radiometer blood gas analyser (model ABL 3) and were maintained within physiological limits ( $P_{O_2}$  > 100 mmHg,  $P_{CO_2}$  > 28–35 mmHg, pH 7.35–7.45). When necessary, arterial  $P_{O_2}$  was increased by enriching the inspired  $O_2$  supply and pH was corrected by administering NaHCO<sub>3</sub> (1 M, i.v.) and/or by adjusting ventilation. Body temperature was maintained at 36–38 °C with a circulating-water heating pad and a heat lamp.

### Experimental protocols

In each experimental protocol we recorded the activity of one afferent per cat.

**Inhibition of xanthine oxidase.** Oxypurinol (Sigma) was dissolved in 10 ml of saline and 20  $\mu$ l of 5 M NaOH. Oxypurinol (10 mg kg<sup>-1</sup>) was infused (at a rate of 2 ml min<sup>-1</sup> i.v.) 10 min before the second period of ischaemia ( $n = 9$ ). In a different group of controls ( $n = 8$ ), myocardial ischaemia was repeated at an interval of 30 min to document that the afferent response was reproducible.

**Production of anti-PMN polyclonal antibody.** Feline PMNs were isolated from whole blood using a modified Percoll density gradient method from Weyrich *et al.* 1993. Centrifugation (Beckman GPR Centrifuge, CA, USA) was done at 4 °C. Cat blood was collected with vacutainer tubes containing 1.5 ml of acid citrate dextrose solution. Anticoagulated blood was mixed (1:1) with Dulbecco's phosphate-buffered saline (PBS, Sigma). After addition of 6% medical grade dextran (1:5, Sigma), erythrocytes were allowed to settle. The upper plasma fraction was pipetted from the erythrocytes and centrifuged at 400 g for 20 min to obtain platelet-rich plasma (PRP). PRP was decanted from the leukocyte pellet and centrifuged at 2500 g for 10 min to obtain platelet-poor plasma (PPP). The leukocyte pellet was washed with modified Hank's balanced salt solution (mHBSS, JRH Biosciences) and centrifuged at 400 g for 10 min. Contaminating erythrocytes were depleted from the leukocyte 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% and 50% Percoll. Starting with the most dense solution, 4 ml of each solution was layered in a 15 ml polypropylene conical centrifuge tube. Leukocytes were resuspended in PBS, then layered on top of the gradient and centrifuged at 1500 g for 40 min. PMNs were collected at the 62–80% interface and washed with PBS. The PMN pellet was resuspended in PBS and checked for the predominance of PMNs with haematoxylin and eosin stain. Finally, the cells were mixed with either Freund's complete or incomplete adjuvant (1:1, Pierce Chemical; Rockford, IL, USA) prior to injection to induce the production of the polyclonal antibody.

The rabbits used for production of the antibody were immunized by subcutaneous injection of PMNs with Freund's complete adjuvant. The rabbits were boosted every 2 weeks with three subsequent injections of the antigen plus incomplete Freund's adjuvant. Two weeks after completion of the immunization series, under 5 mg kg<sup>-1</sup> i.m. oxymorphone and 0.5 mg kg<sup>-1</sup> acepromazine anaesthesia, 10 ml kg<sup>-1</sup> of blood was collected from the rabbit through arterial puncture of the ear and the serum was stored at -70 °C until further processing. Immunoglobulin was precipitated with 80% saturated ammonium sulfate (SAS; Sigma). As described (Drenckhahn *et al.* 1993), antiserum was centrifuged at 3000 g for 30 min at 4 °C. An equal volume of PBS was added to the supernatant. Under constant stirring, an equal volume of 80% SAS was added slowly. The immunoglobulin was precipitated overnight at 4 °C then centrifuged at 3000 g for 30 min at 4 °C. The supernatant was discarded and the immunoglobulin pellet was resuspended with PBS at half the original volume of serum. The immunoglobulin then was dialysed overnight against three changes of 1 l of PBS. Thereafter, the immune serum was stored in aliquots at -70 °C. The rabbit was killed with an overdose of anaesthetic followed by exsanguination.

**Effect of PMNs on cardiac afferent activity.** The polyclonal antibody raised in rabbits immunized with feline PMNs (1 ml kg<sup>-1</sup>) was diluted in 20 ml of saline. Before and during the second period of myocardial ischaemia, which occurred 45 min later, the antibody was infused i.v. at a rate of 0.15–0.4 ml min<sup>-1</sup> ( $n = 7$ ). In another group of animals ( $n = 7$ ), control serum from rabbits was infused at a similar rate before and during the second ischaemia. The protein concentration (spectrophotometer, Perkin-Elmer, Lambda 3B, Ridgefield, CT, USA; UV at 280 nm) of the control serum (rabbit serum, 3 mg ml<sup>-1</sup>) was matched with the serum containing the polyclonal antibody (3 mg ml<sup>-1</sup>).

**Effects of PMN polyclonal antibody after 5-HT<sub>3</sub> receptor blockade.** Tropisetron (300  $\mu\text{g kg}^{-1}$ , i.v.) was administered after locating an afferent ending in the ventricle prior to ischaemia. Then the polyclonal antibody (1 ml  $\text{kg}^{-1}$ ) was diluted in 20 ml of saline and infused i.v. at a rate of 0.15–0.4 ml  $\text{min}^{-1}$  ( $n = 5$ ); after 45 min of infusion, a second period of myocardial ischaemia was induced.

**Measurement of PMN depletion.** To assess the concentration of PMNs before the infusion of antibody, 1 ml of venous blood was collected. During infusion three to four blood samples were collected every 15 min to determine the effectiveness of the antibody in depleting PMNs and its influence on other blood cellular elements. Blood samples were centrifuged (Clay Adams QBC) in capillary tubes (QBC V venous tube) for 5 min. A veterinary haematology analyser QBC reader (Becton Dickinson, NJ, USA) set for the assessment of blood elements in cats was used to measure the content of the granulocytes and other blood cellular elements. The second period of myocardial ischaemia was initiated only after the granulocyte content was reduced to  $< 1 \times 10^9$  cells  $\text{l}^{-1}$ .

**Measurement of myeloperoxidase activity.** Tissue from the ventricular region at risk and the normal ventricular tissue were stored at  $-70^\circ\text{C}$  until assay. Myeloperoxidase (MPO) activity was determined as reported previously (Longhurst *et al.* 1992). Briefly, ice-cold myocardial tissue was minced and homogenized twice (10% w/v) with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA) in 0.05 M  $\text{KH}_2\text{PO}_4$  (pH 6) containing 0.5% hexadecyltrimethyl-ammonium bromide (HTAB) for 15 s. Supernatant (100  $\mu\text{l}$ ) containing the MPO enzyme was added to 1.9 ml of 0.05 M  $\text{KH}_2\text{PO}_4$  (pH 6) containing  $\text{H}_2\text{O}_2$  (0.0005%) and *o*-dianisidine (0.167 mg  $\text{ml}^{-1}$ ). The activity of the MPO supernatant was quantified kinetically on a temperature-controlled (25  $^\circ\text{C}$ ) spectrophotometer (Perkin-Elmer, lambda 3B). Absorbency at 460 nm was recorded for several minutes and the change of

absorbency over 1 min was measured during the linear portion (0.5–1.5 min). Plasma from another group of animals ( $n = 6$ ) was collected to determine the relationship between MPO and the number of PMNs. Using the Percoll density gradient centrifugation method (as noted above), neutrophils were isolated from plasma collected from six animals. After determining the initial PMN concentration with a haemocytometer (Fisher Scientific), serial dilutions were used to achieve PMN concentrations of  $5 \times 10^6$  and  $5 \times 10^5$ , which yielded  $58 \pm 3$  and  $0.6 \pm 0.044 \mu\text{mol H}_2\text{O}_2 \text{ min}^{-1}$ . These values were close to those achieved in the control and antibody treatment conditions.

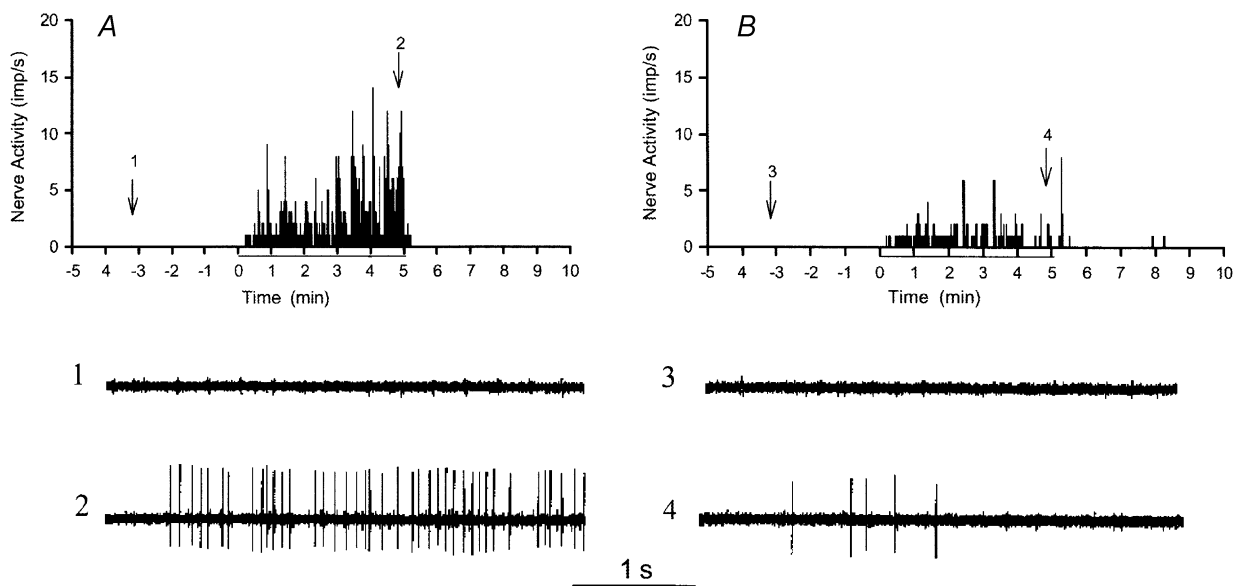
### Data analysis

The discharge frequency of each afferent was averaged (impulses  $\text{s}^{-1}$ ) during 5 min of the pre-ischaemia control period, 5 min of cardiac ischaemia, and the initial 5 min of reperfusion. Data were presented as means  $\pm$  s.e.m. Differences in responses of ischaemically sensitive cardiac afferents to repeated ischaemic stimuli with or without application of oxypurinol or the polyclonal antibody directed against PMNs were analysed by means of repeated measures analysis of variance on ranks, followed, when necessary, by a *post hoc* Student-Newman-Keuls test. Differences were considered to be significant when  $P < 0.05$ . Differences in MPO activity were analysed using Student's paired *t* test. Linear regression was used to determine the relationship between MPO activity and the number of PMNs. Statistical calculations were performed with SigmaStat software (Jandel Scientific Software, San Rafael, CA, USA).

## RESULTS

### Oxypurinol protocol

The effect of oxypurinol as an inhibitor of xanthine oxidase was examined in nine afferents from nine animals. The activity of an ischaemically sensitive cardiac afferent



**Figure 1**

A and B, time stimulus histograms showing the frequency of action potentials (impulses  $\text{s}^{-1}$ ) in a left ventricular afferent during 5 min control, 5 min ischaemia and 5 min reperfusion before and after the administration of oxypurinol, respectively. Neurograms 1 and 2, taken at the times indicated by arrows in A, display the activity before oxypurinol, during control and ischaemia, respectively. Neurograms 3 and 4 display the activity after oxypurinol, during control and ischaemia, respectively, at the times indicated in B.



**Table 1. Influence of rabbit serum, antiserum and PMN antibody on total white blood count, granulocytes, lymphocytes and platelets**

	WBC			Granulocytes (PMNs)			Lymphocytes			Platelets		
	Control	Test	Change (%)	Control	Test	Change (%)	Control	Test	Change (%)	Control	Test	Change (%)
Control serum	15 ± 2.5	16 ± 2.7	3	16 ± 3	16 ± 3.2	4	2.9 ± 0.1	2.7 ± 0.2	7	249 ± 15.5	269 ± 15.8	8
PMN-Ab	12 ± 3.6	2.5 ± 0.5*	79	10 ± 3.5	0.6 ± 0.1*	94	1.7 ± 0.2	2.2 ± 0.5	29	223 ± 17.5	88 ± 10.6*	61
PMN-Ab + tropisetron	18 ± 1.5	2.9 ± 0.3*	84	15 ± 1.8	0.7 ± 0.09*	95	2.7 ± 0.4	2.2 ± 0.2	19	272 ± 20.5	158 ± 21.4*	42

Values represent mean ± S.E.M. ( $10^9$  cells  $l^{-1}$ ). Control and Test represent the first and second periods of ischaemia, respectively. \* Treatment significantly ( $P < 0.05$ ) reduced the cell counts compared to before treatment. WBC, white blood cells; PMNs, polymorphonuclear leukocytes.

innervating the left ventricle before and after infusion of oxypurinol is displayed in Fig. 1. Figure 1A shows the discharge rate of the afferent before treatment with oxypurinol during control, myocardial ischaemia and reperfusion. Oxypurinol reduced the discharge frequency of the ischaemically sensitive cardiac afferent (Fig. 1B). Overall, the responses of the group ( $n = 9$ ) were reduced by an average of 44 % (Fig. 2B).

Repeated myocardial ischaemia, separated by a 30 min period of recovery without treatment, consistently stimulated the cardiac afferents in eight animals (Fig. 2A). We have observed similar reproducible cardiac afferent responses to repeated myocardial ischaemia in previous studies (Pan & Longhurst, 1995; Tjen-A-Looi *et al.* 1998).

### Anti-PMN antibody protocol

The responses of seven ischaemically sensitive afferents were examined before and after administration of the polyclonal antibody (Fig. 3). Figure 3A shows afferent activity before depletion of PMNs during control, myocardial ischaemia and reperfusion. The response of the afferent to ischaemia was decreased by the antibody (Fig. 3B). Responses of the entire group of afferents in response to the antibody were decreased by 66 % (Fig. 4A).

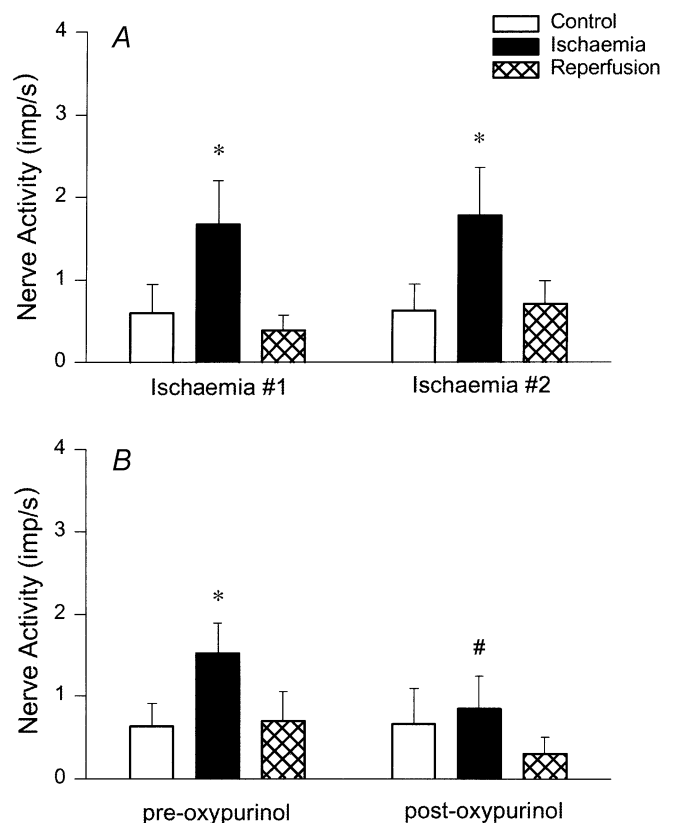
### Effect of PMN antibody on cell counts

The number of circulating PMNs was decreased by 94 % during infusion of the polyclonal antibody (Table 1). Since the concentration of lymphocytes did not change, the decreased numbers of white blood cells (WBCs) reflected a decrease in granulocytes. We also observed a smaller (61 %) decrease in the concentration of platelets. Similar changes in PMNs were observed with the infusion of this antibody after pretreatment with tropisetron (Table 1). Changes in the PMNs and platelets in response to the antibody lasted for more than 1 h ( $P < 0.05$ ). Cell counts did not change in the seven animals infused with control serum (Table 1).

### Tropisetron and anti-PMN antibody protocol

To investigate the significance of a decreased platelet count after infusion of the polyclonal antibody, we

pharmacologically neutralized the potential influence of this cellular component. Thus, in five cats the responses of five ischaemically sensitive cardiac afferents to treatment with the antibody were examined after pretreatment with tropisetron, a selective 5-HT<sub>3</sub> receptor antagonist. In contrast to the influence of the antibody without tropisetron, the response of these afferents to myocardial ischaemia did

**Figure 2**

A, histogram showing group data of eight cardiac afferents yielding consistent responses during repeated ischaemia. B, group data of nine cardiac afferents responsive to ischaemia before and after administration of oxypurinol ( $10 \text{ mg kg}^{-1}$ , i.v.). \* Responses during control and ischaemia were significantly different ( $P < 0.05$ ). # Responses during ischaemia before and after oxypurinol likewise were significantly different ( $P < 0.05$ ).

not change in response to depletion of PMNs and reduction of platelets by the antibody (Fig. 4B), following treatment with tropisetron.

Seven other cardiac afferents in seven cats demonstrated consistent responses before and after the infusion of control serum during myocardial ischaemia (Fig. 5).

### Myocardial MPO activity

Compared to six animals treated with control serum the polyclonal antibody reduced myocardial tissue MPO activity ( $60 \pm 10$  vs.  $10 \pm 1 \mu\text{mol H}_2\text{O}_2 \text{ min}^{-1}$ ). Isolation of neutrophils from plasma in six other animals followed by serial dilution with saline established that these MPO values corresponded to approximately  $5 \times 10^6$  and  $8.9 \times 10^5$  PMNs, control vs. antibody treatment, respectively. Thus, the antibody substantially reduced the amount of neutrophils in the myocardium.

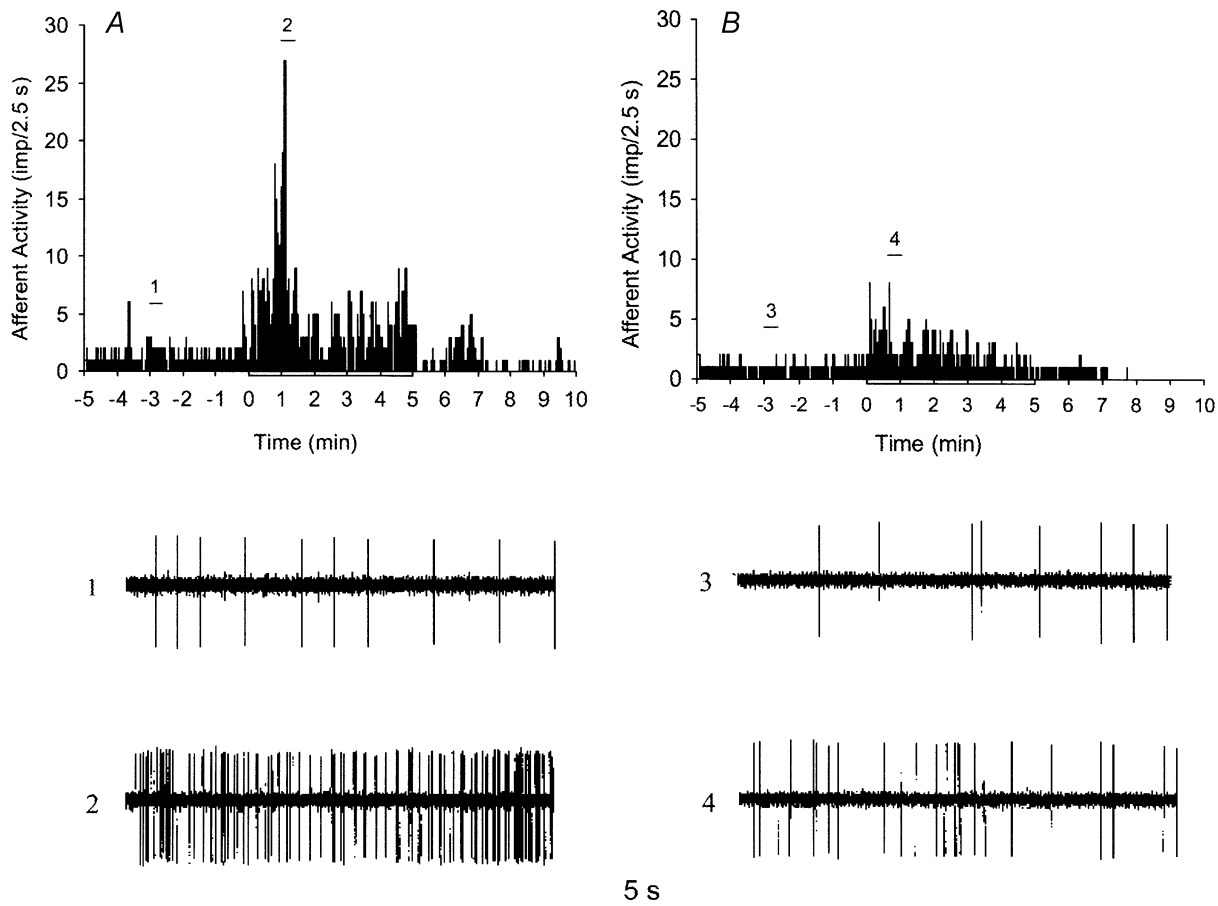
### Receptive fields of cardiac afferents

Most ischaemically sensitive cardiac afferents were located in the left ventricle (Fig. 6). Many were identified to be

near the left circumflex and the left anterior descending coronary vessels. Conduction velocities ranged from 0.33 to  $2.4 \text{ m s}^{-1}$ .

## DISCUSSION

This is the first study to demonstrate that prevention of purine breakdown with an inhibitor of xanthine oxidase significantly influences the responses of cardiac sympathetic afferents during ischaemia. These data suggest that ROS derived from purine degradation are important stimuli of these nerve endings during ischaemia. We also observed a significant reduction in the response of cardiac sympathetic afferents to ischaemia following depletion of circulating and myocardial PMNs with a polyclonal antibody directed against PMNs. To a smaller extent, this antibody also reduced circulatory platelets. Since platelets, through the release of serotonin, can activate cardiac afferents during ischaemia through a 5-HT<sub>3</sub> receptor mechanism (Fu & Longhurst, 2000), we pretreated a group of animals with tropisetron, a 5-HT<sub>3</sub> receptor antagonist. We found that



**Figure 3**

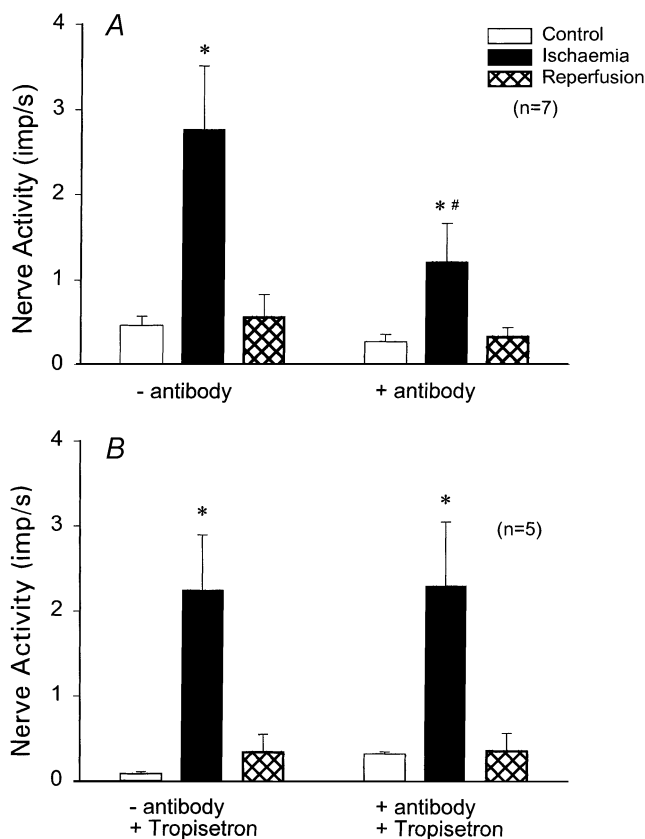
A and B, histograms showing the frequency of action potentials in a representative left ventricular afferent during control, ischaemia, and reperfusion, before and after the administration of antibody, respectively. Neurograms 1 and 2, taken at times indicated by bars in A, display the activity of the afferent before administration of antibody during control and ischaemia, respectively. Neurograms 3 and 4 display the activity of the afferent after antibody during control and ischaemia, respectively, at times shown in B.

pre-blockade of 5-HT<sub>3</sub> receptors with tropisetron prevented the influence of the anti-PMN antibody on the cardiac afferent response to ischaemia. Thus, with respect to potential sources of ROS, purines, but not PMNs, appear to be important in stimulating cardiac sympathetic afferents.

Xanthine oxidase, localized in vascular endothelium, is an important enzyme in health and disease. For example, it promotes iron absorption in the small intestine (Parks & Granger, 1986) and is involved in the pathogenesis of various pathological conditions, including myocardial injury resulting from ischaemia and reperfusion (Simpson & Lucchesi, 1987). Xanthine oxidase plays a major role in the generation of ROS (Kuppusamy & Zweier, 1989; Thompson-Gorman & Zweier, 1990). In reperfused tissue, in the presence of its substrates, hypoxanthine or xanthine, xanthine oxidase reduces molecular oxygen to O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub>, which can react further to form the very reactive species, •OH (Thompson-Gorman & Zweier, 1990; Kuppusamy & Zweier, 1989). Molecular •OH and O<sub>2</sub><sup>•-</sup> free radicals, in turn, oxidize cellular proteins and membranes

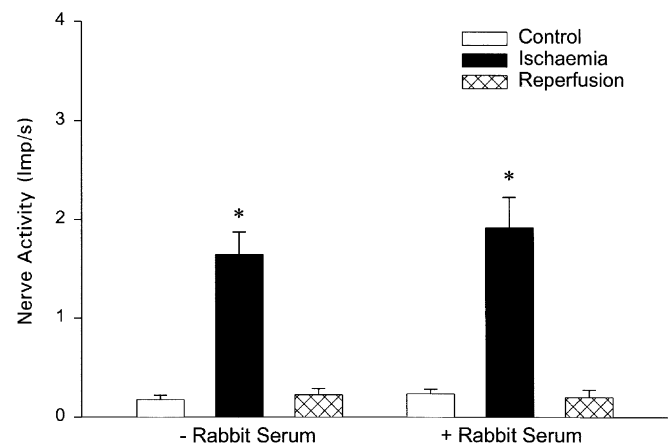
leading to cellular injury (Xia *et al.* 1996). Previous studies have demonstrated that oxypurinol inhibits xanthine oxidase activity (Pourcyrous *et al.* 1993; Zweier *et al.* 1994). In addition, oxypurinol reacts with PMN-generated myeloperoxidase-derived hypochlorous acid at physiological pH, which inactivates human α1-antiprotease. Oxypurinol protects human alpha α1-antiprotease against inactivation by reacting with hypochlorous acid (Grootveld *et al.* 1987) and thereby potentially reduces tissue injury. In ischaemic myocardial tissue, adenosine and its metabolites, inosine and hypoxanthine, are increased in interstitial fluid during brief (5 min) ischaemia (Van Wylen, 1994). In the present study we were able to demonstrate that ROS generated from the breakdown of adenosine contribute to stimulation of cardiac afferents during myocardial ischaemia.

Our study demonstrated that the inhibition of xanthine, and therefore ROS production, reduced the activation of cardiac sympathetic afferents during myocardial ischaemia. Thus, metabolites of adenosine, which can lead to the formation of ROS, play a role in the activation of cardiac sympathetic afferents during myocardial ischaemia. Previous studies by M. H. Huang *et al.* (1995, 1996) reported that adenosine stimulates cardiac sympathetic afferents in dogs (M. H. Huang *et al.* 1995, 1996). Dibner-Dunlap *et al.* (1993) and Thames *et al.* (1993) also suggested that adenosine may activate cardiac sympathetic afferents resulting in reflex sympathoexcitation in dogs (Dibner-Dunlap *et al.* 1993; Thames *et al.* 1993). In contrast, past studies found, through the direct recording of cardiac afferents in cats, that even high concentrations of adenosine and CPA, an adenosine A1 agonist, failed to stimulate ischaemically sensitive cardiac sympathetic afferents in cats during exogenous administration (Pan & Longhurst,



**Figure 4**

A, histograms representing group data of seven cardiac afferents responsive to ischaemia before and after administration of antibody (3 mg ml<sup>-1</sup> kg<sup>-1</sup>, i.v.). B, group data of five cardiac afferents responsive to ischaemia, pretreated with tropisetron, a 5-HT<sub>3</sub> antagonist, before and after administration of antibody. \* Responses occurring during control and ischaemia were significantly different ( $P < 0.05$ ). # Effect of ischaemia before and after antibody was significantly different ( $P < 0.05$ ).



**Figure 5**

Responses of cardiac afferents during ischaemia before and after administration of control serum ( $n = 7$ ). \* Responses during ischaemia were significantly different compared to control ( $P < 0.05$ ) but did not differ between the first and second periods of ischaemia.

1995). We also found that blockade of adenosine receptors with aminophylline did not reduce the response of the cardiac afferents during ischaemia. Similar to our results, Gnecci-Ruscione *et al.* (1995) have shown that aminophylline, an adenosine antagonist, does not alter the response of cardiac afferents during myocardial ischaemia in cats (Gnecci-Ruscione *et al.* 1995). Differences in the experimental design (i.e. reflex *vs.* afferent recording study) and species studied (dog *vs.* cat) may lead to the differential results with respect to the role of adenosine on cardiac afferent nerve endings. Thus, current opinion is divided on the role of adenosine on cardiac afferent endings during ischaemia although there appears to be little evidence in cats that adenosine is an important mediator. Although adenosine is increased during myocardial ischaemia, the present study was not designed to study the role of adenosine in the activation of ischaemically sensitive cardiac fibres. Conversely, it appears from the current data that one of adenosine's metabolites, xanthine, can serve as a source for ROS, which are known to stimulate cardiac sympathetic afferents during ischaemia.

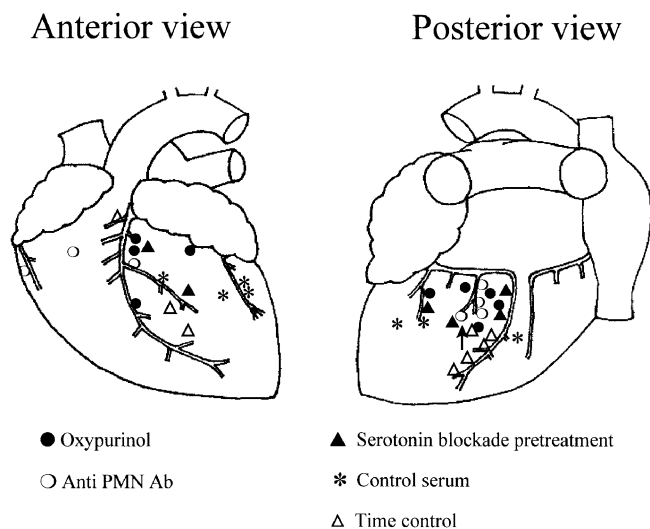
With respect to the influence of PMNs on sensory nerves, Bennett *et al.* (1998) have suggested that thermal hyperalgesia involving activation of somatic afferents, is dependent on the presence of PMNs. Thus, we speculated that these WBCs may serve as a source of ROS, like  $\bullet\text{OH}$  (O'Neill *et al.* 1996), that have the capability of stimulating cardiac afferent nerve endings during myocardial ischaemia and reperfusion.

Myocardial ischaemia activates components of the complement system, which act as chemoattractants to

PMNs leading to their accumulation in the myocardium (Hill & Ward, 1971; McManus *et al.* 1983; Rossen *et al.* 1985; Fletcher *et al.* 1993). Granulocytes are associated with reperfusion injury in ischaemic myocardium. Thus, there is a direct relationship between myocardial infarct size following reperfusion and the extent of infiltration by PMNs (Romson *et al.* 1983; Jolly *et al.* 1986), since PMNs infiltrate the myocardium early after the onset of ischaemic injury (Mallory *et al.* 1939; Fishbein *et al.* 1978; Mullane *et al.* 1985). Inhibition of the function of neutrophils reduces ischaemic damage in the myocardium (Bednar *et al.* 1985). In the present study, using a MPO assay we demonstrated an increase in the number of granulocytes after only 5 min of ischaemia, i.e. before the time required to produce infarction (Jennings & Ganote, 1974). Furthermore, we showed that depletion of PMNs with a polyclonal antibody, as measured by MPO assay, is associated with a reduced discharge frequency of cardiac sympathetic afferents during regional myocardial ischaemia.

Extrapolation of our data suggests that the antibody decreased myocardial PMNs by 82% and circulating PMNs by 94%. However, the antibody also decreased the number of circulating platelets, presumably because the rabbits in which the antibodies were produced were immunized with platelet antigens. Although the 61% decrease in circulating platelets was less than the decreases in circulating and myocardial PMNs, infusion of anti-PMN polyclonal antibody may have influenced platelet activity. In this context, our recent data suggest that platelet activation during brief myocardial ischaemia may serve as a stimulus of cardiac sympathetic afferents through a 5-HT<sub>3</sub> receptor mechanism (Fu & Longhurst, 2000). Thus, to reduce the confounding influence of platelet activation, we pretreated animals with tropisetron, a 5-HT<sub>3</sub> receptor antagonist, a manoeuvre that eliminated the antibody-related reduction of afferent discharge during ischaemia. These data suggest, therefore, that platelets rather than PMNs, are important cellular constituents in the ischaemia-related activation of cardiac sympathetic afferents.

In summary, in addition to the Haber-Weiss reaction, purine metabolism through the xanthine oxidase pathway appears to be an important mechanism in the increased activity of cardiac sympathetic afferents during ischaemia. Conversely, PMNs do not appear to be required for this response. Once activated, these sensory nerves provide signals that ultimately lead to angina pectoris and important sympathoexcitatory cardiovascular reflexes. Such reflexes are deleterious since they can cause cardiac tachyarrhythmias and peripheral vasoconstriction leading to further imbalances between myocardial oxygen supply and demand and, hence, worsen the ischaemic condition. These results amplify our past studies demonstrating that brief ischaemia of the cat heart leads to the production of ROS,



**Figure 6**

Location of receptive fields of ischaemically sensitive cardiac afferents in the left ventricle.  $\Delta$ , repeated ischaemia ( $n = 8$ );  $\bullet$ , oxypurinol ( $n = 9$ );  $\circ$ , antibody ( $n = 7$ );  $\blacktriangle$ , 5-HT<sub>3</sub> antagonist + antibody ( $n = 5$ );  $*$ , rabbit control serum ( $n = 7$ ).



which play an important role in stimulating ischaemically sensitive cardiac sensory nerves.

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