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A novel adenosine analog, AMP579, inhibits neutrophil activation, adherence and neutrophil-mediated injury to coronary vascular endothelium

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Abstract

We hypothesized that 1*S*-[1*a*,2*b*,3*b*,4*a*(*S**)]-4-[7-[[1-[(3-chloro-2-thienyl)methylpropyl]propyl-amino]-3*H*-imidazo[4,5-*b*] pyridyl-3-yl]-*N*-ethyl-2,3-dihydroxycyclopentane carboxamide (AMP579), a new adenosine analog, inhibits superoxide anion (O_2^-) generation and degranulation from canine neutrophils, neutrophil adherence and neutrophil-induced dysfunction to canine coronary artery endothelium by adenosine receptor-mediated mechanisms. AMP579 inhibited O_2^- generation (nM/20×10^6 neutrophils) from platelet activating factor (PAF)-activated neutrophil in concentration-dependent manner (4.1 ± 0.8 at 10 μM vs. 16.7 ± 2.1 in PAF group, $P < 0.05$). This inhibitory effect was blocked by the adenosine A_{2A} receptor-selective antagonist, 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385, 17.7 ± 2.8, $P < 0.05$), but not by either the adenosine A_1 receptor-selective antagonist, 8-Cyclopentyl-1.3-dipropylxanthine (DPCPX) or the adenosine A_3 receptor-selective antagonist, 9-Chloro-2-(2-furanyl)-5-[(phenylacetyl)amino][1,2,4]-triazolo[1,5-*c*]quinazoline (MRS1220). AMP579 inhibited neutrophil degranulation dose-dependently by $38 \pm 2\%$ at 10 μM ($P < 0.05$). The inhibitory effect of AMP579 was not altered by either DPCPX or MRS1220, but was partially reversed by ZM241385 ($69 \pm 8\%$, $P < 0.05$ vs. AMP579 10 μM). A total of 10 μM AMP579 reduced neutrophil adherence to thrombin-stimulated endothelium (neutrophils/mm^2) from 269 ± 16 to 44 ± 4 ($P < 0.05$); this was reversed by ZM241385, but not by DPCPX or MRS1220. After coincubation of unstimulated neutrophil with thrombin-stimulated endothelium, concentration–relaxation responses to the endothelium receptor-dependent vasodilator, acetylcholine, were reduced (maximum $57 \pm 5\%$ vs. $120 \pm 5\%$ in controls, $P < 0.05$). This endothelial dysfunction was attenuated by AMP579 ($116 \pm 7\%$, $P < 0.05$). We conclude that AMP579 inhibits neutrophil activation and neutrophil-mediated coronary endothelial dysfunction, primarily by an adenosine A_{2A} receptor mechanism.

Keywords: AMP579; Coronary artery endothelium; Neutrophil degranulation; Neutrophil adherence; Superoxide anion

1. Introduction

It is well known that the coronary vascular endothelium plays a pivotal role in the regulation of vasomotor tone, maintenance of myocardial blood flow, and attenuation of intravascular platelet aggregation and neutrophil adherence to the vascular endothelium (Becker and Ambrosio, 1987; Hearse et al., 1993; Brutsaert et al., 1998; Cotran et al., 1999). These actions are partially due to endothelial secretion of a variety of vasoactive substances such as adenosine, prostaglandin, and endothelium-derived relaxing factor. In addition, the endothelium is also a target for injury at different pathophysiological conditions. For example, activation of blood cells, especially neutrophils, and cell–cell interactions between neutrophil and vascular endothelial cells have been linked with myocardial ischemia–reperfusion injury. Release of proinflammatory mediators

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such as cytokines, superoxide anion, and myeloperoxidase through neutrophil degranulation has been reported (Becker and Ambrosio, 1987; Forman et al., 1989; Lucchesi et al., 1989; Verrier and Boyle, 1996).

Adenosine is an endogenous autacoid released during myocardial ischemia that has been shown to exert protective effects by preventing neutrophil activation, neutrophil adherence, and neutrophil-induced injury to coronary artery endothelium (Cronstein et al., 1986; Zhao et al., 1996). These beneficial effects of adenosine have been associated with its cardioprotection during ischemia and reperfusion (Olafsson et al., 1987; Todd et al., 1996; Jordan et al., 1997). However, the short half-life of adenosine (a few seconds) and a necessity for higher concentrations for inhibition of neutrophil and endothelial cell interactions may limit its cardioprotective effect in in vivo situation (Cronstein et al., 1986; Zhao et al., 1996). Therefore, analogs with effective anti-neutrophil activation at lower concentrations would be desirable.

1*S*-[1*a*,2*b*,3*b*,4*a*(S^*)]-4-[7-[[1-[(3-chloro-2-thienyl)methylpropyl]propyl-amino]-3*H*-imidazo[4,5-*b*] pyridyl-3-yl]-*N*-ethyl-2,3-dihydroxycyclopentane carboxamide (AMP-579), is an adenosine analog with an affinity ($K_i = 5$ nM) for the adenosine A_1 receptor and an affinity ($K_i = 56$ nM) for adenosine A_{2A} receptor. Selected doses of AMP579 at lower concentrations relative to adenosine have been shown to be cardioprotective when administered either during ischemia or prior to reperfusion (Smits et al., 1998; McVey et al., 1999). However, the effect of AMP579 on neutrophil activation and cell–cell interactions between neutrophil and vascular endothelial cell has not been investigated. Therefore, the present study tested the hypotheses that AMP579 (1) directly inhibits superoxide anion generation from neutrophils activated by platelet activating factor (PAF), (2) inhibits release of myeloperoxidase through degranulation of PAF-activated neutrophils, (3) reduces the adherence of unstimulated neutrophils to activated coronary artery endothelium, and (4) attenuates neutrophil-induced dysfunction to the coronary artery endothelium.

2. Materials and methods

2.1. Surgical preparation of animals

All animals received humane care in compliance with the ''Principles of Laboratory Animal Care'' formulated by the National Society for Medical Research and ''Guide for the Care and Use of Laboratory Animals'' proposed by the National Institutes of Health (NIH Publication No. 80-23, revised 1985).

Dogs of either sex weighing between 21.2 and 30.9 kg were used in the study. All animals were initially anesthetized with intravenous 2% sodium pentothal (20 mg/kg). Each dog was endotracheally intubated and ventilated with oxygen-enriched room air to maintain arterial oxygen tension greater than 100 mm Hg with use of a volume-cycled respirator. Peripheral blood was collected from the femoral artery for isolation of neutrophils. A median sternotomy was performed, and the pericardium was widely opened. After blood collection and subsequent heparinization (300 U/kg), additional sodium pentobarbital (10 mg/kg) was injected for sacrifice. The heart was then excised and immediately immersed in cold oxygenated Krebs–Henseleit (K–H) buffer having the following compositions (mM): 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5$CaCl_2$, 12.5 $NaHCO_3$, and 10 glucose. The left anterior descending coronary artery and circumflex coronary artery were carefully isolated from the heart so as not to disturb the endothelium. Isolated coronary vessels were cleaned of adipose and connective tissue and cut into rings approximately 3–4 mm in length.

2.2. Neutrophil isolation

Peripheral blood (200 ml for each sample) was collected and mixed with 45 ml of anticoagulating agents and 100 ml 6% dextran, which include 1.6% citric acid and 2.5% sodium citrate. After the erythrocytes were sedimented (50 min at room temperature), the leukocyte-rich plasma layer was removed into a 50-ml plastic centrifuge tube and centrifuged at $400 \times g$ for 10 min at 4°C. Any contaminating erythrocytes in the pellet were removed by hypotonic lysis for 20 s with 9 ml of sterile distilled water. Subsequent addition of 3 ml of 0.6 mol KCl and 25 ml of buffered Hanks' balanced salt solution (HBSS) rapidly returned this solution to isotonicity. The leukocyte-rich suspension was centrifuged at $400 \times g$, and cells were resuspended in 2 ml of HBSS, then layered on top of 3 ml of Ficoll–Pacque (Sigma, St. Louis, MO), and centrifuged at $600 \times g$ for 20 min. The resulting pellet was rinsed with Ca^{2+}- and Mg^{2+}-free HBSS, and the cells were counted. Final suspensions contained 94% neutrophils, and cell viability was 99% as determined by trypan blue exclusion (Zhao et al., 1996).

2.3. Superoxide anion generation by neutrophils

Superoxide anion generation by canine neutrophils in suspension was determined by measuring the superoxide dismutase-inhibitable reduction of ferricytochrome *c* to ferrocytochrome *c*. Neutrophils (20×10^6 cells/ml) were prewarmed (37°C) in 160 μM cytochrome *c* and incubated in the presence of cytochalasin B (5 μg/ml) and test agents under consideration (e.g., AMP579 and adenosine receptor antagonists) at designated concentrations for 5 min. A total of 5 μM of PAF (determined by pilot studies) was used to stimulate canine neutrophils. The final reaction volume was 0.5 ml. Superoxide dismutase (100

μg/ml) was added to parallel samples. The difference between ferrocytochrome *c* formation in the presence and absence of an excess of superoxide dismutase was used to determine the superoxide anion generation. After 5 min of incubation, the reaction was stopped by placing the tubes on ice and centrifuging at $500 \times g$ and 4°C for 10 min. Ferrocytochrome *c* formation was measured spectrophotometrically by determining the optical density of the supernatant at a wavelength of 550 nm, using a SpectroMax UV-Vis Plate Reader (Molecular Devices, Palo Alto, CA). The results were reported as nanomolars of superoxide anion generation per 20×10^6 neutrophils per 5 min (Zhao et al., 1996).

2.4. Neutrophil degranulation

Supernatant myeloperoxidase activity was measured as the product of neutrophil degranulation using a modification of the method by Ely et al. (1995). Neutrophils (20×10^6 cells/ml) were incubated in the presence or absence of different concentrations of AMP579 for 5 min at 37°C. The cells were then incubated with PAF (final concentration 5 μM) and cytochalasin B (5 μg/ml). After being incubated at 37°C for 10 min and centrifuged ($500 \times g$, 15 min), the cell-free supernatant was mixed in a 1:4 ratio with myeloperoxidase solution (6 ml 0.01 M potassium phosphate, 100 μl *O*-dianisidine (100 mg/ml), and 60 μl hydrogen peroxide (0.3%)). Myeloperoxidase activity of the resulting supernatants were immediately measured on a SpectroMax UV-Vis Plate Reader (Molecular Devices) at a wavelength of 450 nm for 5 min. Results were expressed as a percent of PAF-stimulated neutrophil myeloperoxidase activity.

2.5. Neutrophil adherence assay

Alterations in the adherence component of neutrophil activity by AMP579 were assessed with unactivated neutrophils labeled with a vital fluorescent dye as described previously (Zhao et al., 1996). Diluent (1.0 ml) and PKH26-GL dye (1.0 ml, 4 μM) were added to the neutrophil suspension ($< 10^7$ cells) and then mixed for 5 min. Phosphate-buffered saline (PBS, 2.0 ml) containing 10% plasma was subsequently added to stop the reaction. Cells were then centrifuged at $600 \times g$ for 10 min at 4°C. The pellet was resuspended in PBS, and the number of cells was counted. The labeling procedure yielded cells possessing normal morphology and function. Coronary rings, 3–4 mm in length, were carefully opened into segments without disturbing the endothelium and placed in 5 ml round cell culture dishes containing 2 ml of K–H buffer at 37°C. Coincubation of AMP579 and adenosine receptor antagonists at the indicated concentration with coronary arteries was started 5 min before thrombin stimulation. Incubation of thrombin (2 U/ml) with segments for 20 min was used to activate endothelium. Fluorescently labeled neutrophils (1.6×10^6 cells/ml) were then added to the baths after thrombin was washed out, and allowed to incubate further for an additional 20 min. After incubation, coronary artery segments were removed and dipped three times in fresh K–H solution to remove non-adherent neutrophils, placed on a glass slide with immersion oil, and covered with a cover glass. Adherence was determined by counting the number of neutrophils adhered to the endothelial surface per square millimeter of the endothelium in six separate microscopic fields under epifluorescent microscopy (rhodamine filter cube, Olympus). The observer was blinded to the group assignment until all slides from our experiment were observed and counted.

2.6. Neutrophil-mediated coronary artery endothelial dysfunction

Coronary artery rings of 3–4 mm in length were mounted on stainless steel hooks and placed into Radnoti organ chambers (Radnoti Glass, Monrovia, CA) containing 7 ml of oxygenated (95% O_2–5% CO_2) K–H solution at 37°C. The rings were then mounted on stainless-steel hooks and connected to FT-03 force displacement transducers (model TR001, Radnoti). Changes in isometric force were digitized at 2 Hz by using an analog-to-digital converter and were analyzed with a windows-based videographics program on PC computer (SPECTRUM, Wake Forest University, Winston-Salem, NC). The optimal baseline force that gave maximal constrictor responses to 30 mM KCl was used as the initial prestretch tension. This initial tension was used in subsequent procedures. After 60 min of equilibration, coincubation of AMP579 and adenosine receptor antagonists with coronary artery rings was started 5 min before addition of thrombin (2 U/ml). After 20 min stimulation of rings, thrombin was washed out and neutrophils (40×10^6 cells/ml) were then added to the baths, and allowed to incubate further for an additional 40 min under 0 g tension. The coronary rings were subsequently washed to remove neutrophils and were placed at the initial prestretch tension by progressively stretching. Indomethacin (10 μM) was added during the last 20 min of incubation and thereafter to inhibit release of prostaglandin. As we reported previously, (Sato et al., 1996) dose–response curves to the thromboxane A_2 mimetic agent, U46619, were performed in all rings to determine the optimal concentration of U46619 for precontraction. A total of 5 nM of U46619 was finally used for precontraction of all rings. Endothelial function was assessed by comparing vasorelaxation to incremental concentrations of the endothelium-dependent vasodilator, acetylcholine (1–186 nM). After recording the response to acetylcholine, the rings were washed several times and allowed to equilibrate to baseline tone. The procedure was repeated for the

endothelium-independent vasodilator, sodium nitroprusside (1–381 nM). Drug concentrations were expressed as final concentrations in the organ chamber.

2.7. Chemicals

The following drugs were purchased from Sigma: acetylcholine chloride, nitroprusside, adenosine deaminase, superoxide dismutase (3000 U/mg, from bovine erythrocytes), cytochalasin B, ferricytochrome c (horse heart type VI), indomethacin, thrombin (from human plasma), and PKH26-GL. Thromboxane A_2 mimic U46619 was donated from Upjohn (Kalamazoo, MI). 8-Cyclopentyl-1.3-dipropylxanthine (DPCPX) and 9-Chloro-2-(2-furanyl)-5-[(phenylacetyl)amino][1,2,4]-triazolo[1,5-*c*]quinazoline (MRS1220) was purchased from Research Biochemicals (Natick, MA). 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385) was purchased from Tocris Cookson (Ballwin, MO). PAF was purchased from Biomol (Plymouth Meeting, PA).

2.8. Statistical analysis

All data are presented as the mean ± S.E.M. On superoxide anion generation, neutrophil degranulation, and neutrophil adherence assay, IC_{50} was graphically calculated from concentration–response curves independently and expressed as micromolars of the drug concentration. Concentration–response curves of vascular relaxation are calculated as a percentage of the decrease of U46619-induced isometric constrictor force. Graphic determination of the concentration of drug required to elicit 50% of maximal relaxation (IC_{50}) was obtained from concentration–response curves and expressed as a percent of the negative log of the drug concentration ($-\log$ [M]) by using a Windows-based videographics program (SPECTRUM, Wake Forest University). Statistical analysis was performed by a one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. Differences were considered significant if $P < 0.05$.

3. Results

3.1. Superoxide anion generation from activated neutrophils

The concentration-dependent inhibition of superoxide anion generation from canine neutrophils by AMP579 is shown in Fig. 1A, 10 μM of AMP579 resulted in 75% inhibition of superoxide anion generation relative to PAF-

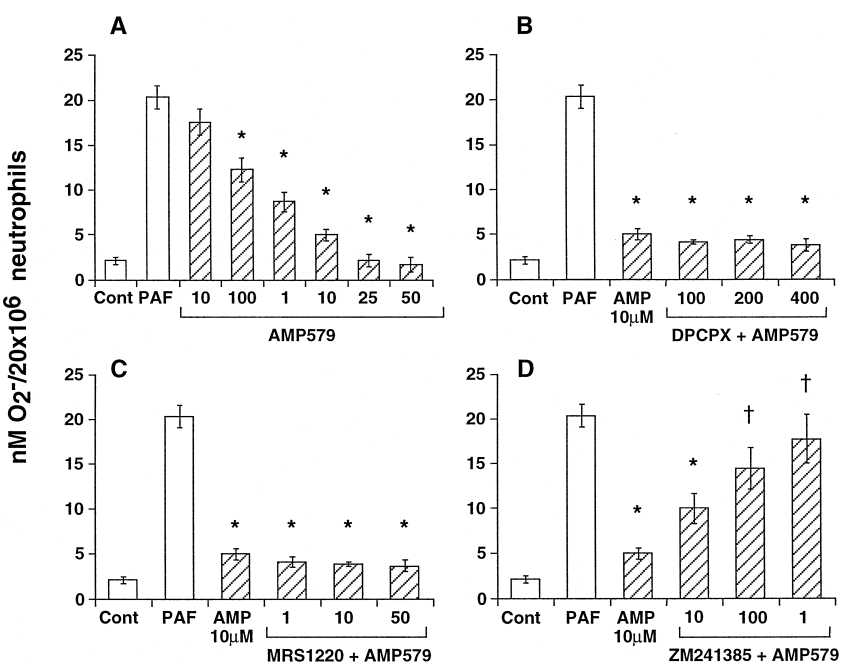

Fig. 1. Graph showing concentration–response of AMP579 (10 nM–50 μM, A) and the effects DPCPX (100–400 nM, B), MRS1220 (1–50 nM, C), and ZM241385 (10 nM–1 μM, D) in the presence of 10 μM of AMP579 on superoxide anion generation from canine neutrophils (20×10^6 cells/ml) stimulated by PAF (5 μM). Data are presented as nanomoles of superoxide anion produced. Cont, unstimulated neutrophils. Values are means ± S.E.M. of at least six separate experiments with duplicate determinations for each experiment. * $P < 0.05$ vs. PAF-stimulated neutrophil group. † $P < 0.05$ vs. 10 μM AMP579 (AMP) group.

stimulated neutrophil with 100% inhibition (to control levels) observed at 25 μM. Calculated IC_{50} of AMP579 on superoxide anion generation was 0.64 ± 0.1 μM. To determine the adenosine receptor subtype involved, the effects of the adenosine A_1 receptor antagonist, DPCPX, adenosine A_3 receptor antagonist, MRS1220, and adenosine A_{2A} receptor antagonist ZM241385 on superoxide anion generation were determined in the presence of a fixed 10 μM of AMP579 concentration. Neither DPCPX at concentrations ranging from 100 to 400 nM (in the range to only block A_1 receptor, Fig. 1B) or MRS1220 from 1 to 50 nM (block A_3 receptor, Fig. 1C) altered the inhibitory effect of AMP579. ZM241385 at concentrations of 100 nM (14.4 ± 2.3 nM) and 1 μM (17.7 ± 2.7 nM) completely counteracted the effect of 10 μM AMP on superoxide anion generation in a concentration-dependent manner (Fig. 1D). These data suggested that the effect of AMP579 on superoxide anion generation primarily involves an adenosine A_{2A} receptor-mediated mechanism. To confirm whether endogenous adenosine released from neutrophils interferes to accurately estimate IC_{50} of AMP579, adenosine deaminase was added into neutrophil suspensions 5 min before PAF stimulation. At effective concentration of 2 units/ml (one unit deaminates 1 μM of adenosine to inosine per minute), adenosine deaminase did not alter superoxide anion generation from PAF-stimulated neutrophils (19.8 ± 1.9 vs. 20.3 ± 1.3 nM, $P > 0.05$).

3.2. Neutrophil degranulation (release of myeloperoxidase)

Inhibition of neutrophil degranulation by AMP579 is shown in Fig. 2A. AMP579 significantly inhibited degranulation from PAF-stimulated neutrophils in a concentration-dependent manner, with a peak at 50 μM (5 ± 1%). IC_{50} of AMP579 on neutrophil degranulation was 7.57 ± 1.84 μM. To determine the adenosine receptor subtype involved, the effects of DPCPX, MRS1220, and ZM241385 on degranulation were determined in the presence of a fixed AMP579 concentration (i.e., 10 μM). The inhibition of AMP579 was not blocked by either DPCPX (Fig. 2B) or MRS1220 (Fig. 2C). However, ZM241385 at the concentrations ranging from 100 nM (63 ± 8%) to 1 μM (69 ± 8%) partially blocked the inhibitory effects of 10 μM AMP579 ($P < 0.05$, Fig. 2D), suggesting that the inhibition of PAF-stimulated neutrophil degranulation by AMP579 may in part involve an adenosine A_{2A} receptor-mediated mechanism.

3.3. Neutrophil adherence to coronary artery endothelium

When unstimulated neutrophils were added to the baths and incubated with unstimulated coronary artery segments for 20 min, an average of 21 ± 2 neutrophils/mm^2 was

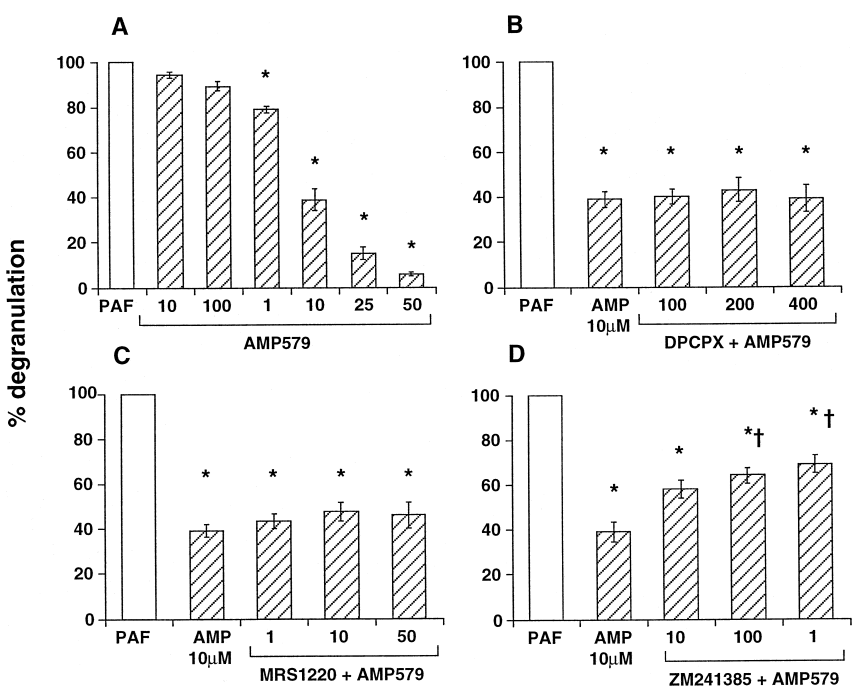

Fig. 2. Graph showing concentration–response of AMP579 (10 nM–50 μM, A) and the effects DPCPX (100–400 nM, B), MRS1220 (1–50 nM, C), and ZM241385 (10 nM–1 μM, D) in the presence of 10 μM of AMP579 on myeloperoxidase release from PAF (5 μM)-stimulated neutrophils (20×10^6 cells/ml). Data are presented as percent of myeloperoxidase release by paired PAF-stimulated canine neutrophil. Values are means ± S.E.M. of at least four separate experiments with duplicate determinations for each experiment. * $P < 0.05$ vs. PAF-stimulated neutrophil group. † $P < 0.05$ vs. 10 μM AMP579 (AMP) group.

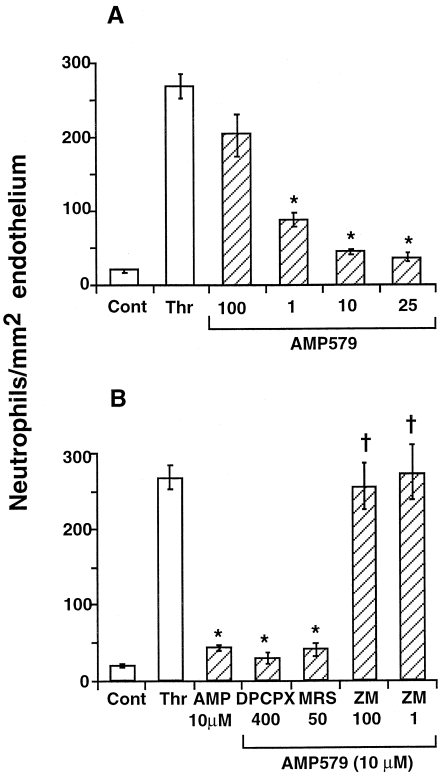

Fig. 3. Graph showing concentration–response of AMP579 (100 nM–25 μM), (A) and the effects DPCPX (400 nM), MRS1220 (MRS, 50 nM), and ZM241385 (ZM, 100 nM and 1 μM) in the presence of 10 μM AMP579 on adherence of fluorescent neutrophil to thrombin (Thr, 2 U/ml)-stimulated coronary artery endothelium (B). Coronary segments were exposed to thrombin, and unstimulated neutrophils were then added for 20 min in the presence of AMP579. Results are presented as number of neutrophil per square millimeter of coronary endothelium. Bar heights represent means ± S.E.M. of at least seven coronary rings from three dogs. $^{*}P < 0.05$ compared with thrombin-stimulated neutrophil group. †$P < 0.05$ vs. 10 μM AMP579 (AMP) group.

observed to adhere to the endothelial surface. Most of the neutrophils maintained a spherical conformation and adhered to the endothelial surface without homotypic aggregation. Incubation of coronary artery segments with thrombin (2 U/ml) resulted in a significant increase in adherence of 269 ± 16 neutrophils/mm^2 ($P < 0.05$ vs. unstimulated). A concentration-dependent inhibition of neutrophil adherence to thrombin-stimulated coronary artery endothelium with AMP579 is shown in Fig. 3A. Coronary artery segments preincubated with 10 μM AMP579 for 5 min before thrombin stimulation significantly inhibited adherence to the endothelium (44 ± 4 neutrophils/mm^2) by 84% compared to thrombin-stimulated coronary artery segments ($p < 0.05$). IC_{50} for inhibition of neutrophil adherence averaged 0.61 ± 0.07 μM. This attenuation of neutrophil adherence by AMP579 was completely reversed with ZM241385 (at 100 nM and 1 μM), but was not altered by either DPCPX (400 nM) or MRS1220 (50 nM), suggesting that inhibition of neutrophil adherence by AMP579 may be mediated by activation of adenosine A_{2A} receptor with little involvement of adenosine A_1 or A_3 receptor mechanism (Fig. 3B).

To further confirm that increased neutrophil adherence on endothelium after thrombin incubation was caused by activated endothelium, the effect of thrombin on superoxide anion generation by neutrophils was observed. As shown in Fig. 4, incubation of neutrophils with thrombin (0.5–4 U/ml) did not show any activating effect, indicating that the effect of thrombin on neutrophil adherence may be induced by direct stimulation of the endothelium.

3.4. Neutrophil-mediated injury to coronary artery endothelium

3.4.1. Endothelium-dependent coronary artery relaxation

Coincubation of thrombin-stimulated coronary segments with unstimulated neutrophils for 40 min significantly attenuated agonist-stimulated endothelium-dependent relaxation. Although the difference in EC_{50} between control rings and rings coincubated with thrombin plus neutrophil did not reach significance (-7.6 ± 0.08 vs. -7.3 ± 0.13 log[M], $P > 0.05$), rings incubated with thrombin plus neutrophil showed a significant decrease in the maximal relaxation responses compared to control rings, ($57 \pm 5\%$ vs. $120 \pm 5\%$ $P < 0.05$, Fig. 5A). Thrombin only had no direct effect on vascular relaxation to acetylcholine in the absence of neutrophils. Incubation of AMP579 with coronary artery segments before addition of thrombin and neutrophils significantly preserved neutrophil-mediated damage to coronary endothelium with a greater maximal relaxation ($116 \pm 7\%$, $P < 0.05$ vs. neutrophil plus thrombin). Since thrombin had no direct effect on neutrophil superoxide anion generation, these data suggest that the protective effect of AMP579 on endothelial dysfunction is mainly exerted by inhibiting neutrophil adherence-dependent pathway.

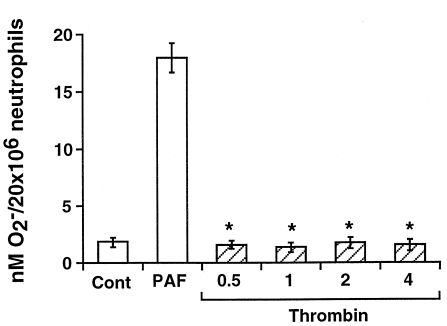

Fig. 4. Graph showing the effect of thrombin on superoxide anion generation from neutrophils. Thrombin (0.5–4 U/ml) did not show any stimulating effect on generation of superoxide anion from neutrophils. Values are means ± S.E.M. of at least four separate experiments with duplicate determinations for each experiment. $^{*}P < 0.05$ vs. PAF-stimulated neutrophil group.

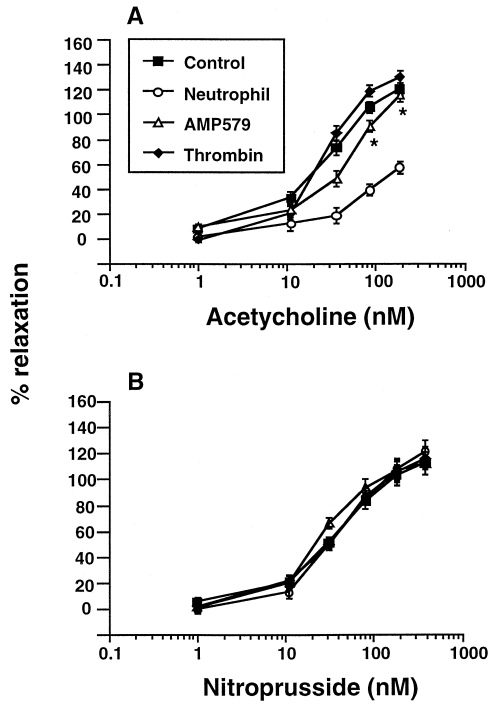

Fig. 5. Effect of AMP579 on neutrophil-mediated dysfunction of agonist-stimulated endothelium-dependent relaxation (A) and endothelium-independent relaxation (B) responses of coronary artery rings. Control: normal rings unexposed to neutrophil and thrombin; Neutrophil: neutrophil plus thrombin-stimulated rings; AMP579: incubation of 10 μM AMP579 with rings before addition of thrombin and neutrophil; Thrombin: thrombin with rings only. Values are means ± S.E.M. of at least seven rings from five dogs. $^{*}P < 0.05$ compared with neutrophil plus thrombin-stimulated rings.

3.4.2. Endothelium-independent coronary artery relaxation

Incubation of thrombin-stimulated coronary artery segments with neutrophil did not cause any damage on endothelium-independent relaxation response to the smooth muscle vasodilator, nitroprusside (Fig. 5B).

4. Discussion

In the present study, we found that AMP579 exerted adenosine A_{2A} receptor-mediated effects including (a) inhibition of superoxide anion generation, (b) attenuation of neutrophil degranulation, and (c) reduction of neutrophil adhesion and neutrophil-induced dysfunction to coronary artery endothelium. Since thrombin had no direct stimulating effect on neutrophil, the alterations in neutrophil adherence to thrombin-stimulated endothelium and attenuation of neutrophil-mediated vascular endothelial dysfunction by AMP579 may suggest that the inhibitory actions of AMP579 are mainly exerted on the endothelium thus preventing adherence and activation of neutrophil. These studies suggest that AMP579 has effective inhibitory effect either on adherence-independent neutrophil activation or adherence-dependent neutrophil-induced vascular dysfunction.

The effect of AMP579 on canine neutrophil activation in the present study was confirmed by inhibiting superoxide anion generation and myeloperoxidase release. Although neutrophil activation may have been inhibited at a range of AMP579 from 100 nM to 25 μM, as supported by our concentration–inhibition data, we chose to use a concentration of 10 μM AMP579 since 75% inhibition of neutrophil activation was achieved at this concentration. The inhibition of superoxide anion generation from PAF-stimulated neutrophils by 10 μM of AMP579 was more effective as compared to a previous report that superoxide anion production from *N*-formyl–L-methionyl–L-leucyl–L-phenylalanine (fMLP)-activated human neutrophils was attenuated with 10 μM of adenosine (less than 50% inhibition) (Cronstein et al., 1983). The inhibitory effect of AMP579 on superoxide anion generation via adenosine A_{2A} receptor mechanism is consistent with other findings using adenosine receptor-specific agonists and antagonists (Cronstein et al., 1985; Roberts et al., 1985; Zhao et al., 1996; Jordan et al., 1997). In the present study, DPCPX and MRS1220 at their highest concentrations for adenosine A_1- and A_3-selective receptors, respectively, did not show any blocking effect on superoxide anion generation by AMP579, favoring an exclusion of the roles of adenosine A_1 and A_3 receptors in inhibition of AMP579 on neutrophil oxidative burst activity. Furthermore, the absence of blocking effect of MRS1220 on superoxide anion generation is consistent with a recent report from our laboratory that a highly selective adenosine A_3 receptor agonist, 2-chloro-N6-(3-iodobenzyl) adenosine-5′-*N*-methyluronamide (CI-IB-MECA) did not alter superoxide anion generation from PAF-stimulated canine neutrophils (Jordan et al., 1998, 1999).

The inhibitory effect of adenosine and adenosine analogs on neutrophil degranulation is not consistently observed. Cronstein et al. demonstrated that both adenosine and an adenosine A_2 receptor agonist reduced the release of β-glucuronidase, a marker of neutrophil degranulation from fMLP-stimulated human neutrophils by only 20% (Cronstein et al., 1988). Bouma et al. found that adenosine inhibited the release of the azurophilic granule proteins bactericidal/permeability-increasing protein and elastase from tumor necrosis factor-α activated human neutrophils by 70–80%. In addition, the inhibition of human neutrophil degranulation by adenosine was partially blocked by an adenosine non-selective receptor antagonist, but not by an adenosine A_1 receptor antagonist, suggesting that the adenosine A_2 and A_3 receptors may be involved (Bouma et al., 1997). However, a recent report from our laboratory (Jordan et al., 1999) showed that degranulation from PAF-stimulated canine neutrophils was not inhibited by the adenosine A_3-selective receptor agonist, CI-IB-MECA, suggesting that the adenosine A_3 receptor may not

be involved in the inhibition of myeloperoxidase released from canine neutrophils. In the present study, AMP579 significantly reduced myeloperoxidase release from PAF-stimulated neutrophils with a peak of 94% inhibition at the highest concentration used (50 μM). The adenosine A_{2A}-selective receptor antagonist, ZM241385, did not counteract the inhibitory effect of AMP579 on neutrophil degranulation completely, suggesting that there may be alternative mechanisms that may be involved in inhibiting degranulation by AMP579 independent of purinergic receptors by Gs protein transduction mechanisms. In this regard, it was previously shown that the activation of inositol 1,4,5-triphosphate (IP_3) pathway to induce the rapid transmembrane Ca^{2+} influx has been linked with neutrophil degranulation (Cronstein, 1994; Cronstein et al., 1988). It has been reported, however, that adenosine has no direct effect on the IP_3 mediated rapid-onset Ca^{2+} transient (Walker et al., 1990; Zhang et al., 1996), and adenosine therefore is unlikely to affect degranulation via an effect on the IP3 pathway. In contrast to adenosine, the concentration-dependent inhibition of neutrophil degranulation by AMP579 suggests that the analog may have the capacity to inhibit the IP_3 pathway as well as the G-protein-mediated adenylate cyclase-dependent receptor pathways. This hypothesis, however, was not confirmed in the present study. Further studies such as IP_3 production or intracellular Ca^{2+} concentration are needed to elucidate the exact mechanism, by which AMP579 attenuated degranulation and release of myeloperoxidase.

We and other investigators have reported that adenosine and adenosine analogs attenuated adherence of PAF-activated canine neutrophil to coronary artery endothelium as well as fMLP-activated neutrophils to cultured human endothelial cells (Cronstein et al., 1983; Zhao et al., 1996). In these studies, both neutrophil and coronary artery endothelium were exposed to PAF and fMLP, thereby stimulating both components. In order to compare with the inhibitory effect of AMP579 on neutrophil activation and endothelial injury, in the present study, we stimulated neutrophil and coronary artery endothelium with PAF and thrombin separately. It has been reported that thrombin increases the surface expression of *P*-selectin on the endothelium, which is the initial ''rolling'' step of neutrophil adherence to the vascular endothelium (Nolte et al., 1994; Lefer et al., 1997). This initial step is prerequisite to cause activation and adherence of neutrophils to endothelium. The present study demonstrated that AMP579 significantly reduced unstimulated neutrophil adherence, suggesting a direct protective effect of AMP579 on the endothelium by preventing thrombin stimulated endothelial activation. Inhibition of AMP579 on neutrophil adherence was counteracted by an adenosine A_{2A} receptor antagonist but not varied by either adenosine A_1 or A_3 receptor antagonist, illustrating adenosine A_{2A} receptor-mediated mechanism and little involvement of adenosine A_1 or A_3 receptor. A potential mechanism for inhibition of neutrophil adherence by AMP579 may involve a modulation of adhesion molecule-mediated interaction between the neutrophils and the endothelial cells. Further studies are required to clarify the mechanisms underlying AMP579's inhibitory effect of adhesion molecules on the endothelium after thrombin stimulation.

In the present study, AMP579 significantly inhibited neutrophil-induced dysfunction to endothelium-dependent vascular relaxation when unstimulated neutrophils were incubated with thrombin-activated coronary artery segments. This injury may largely be caused by superoxide anion, hypochlorous acid and protease released from neutrophils adhered on the vascular endothelium (Lucchesi et al., 1989; Hearse et al., 1993; Verrier and Boyle, 1996; Zhao et al., 1996). In the absence of neutrophils, coincubation of thrombin with coronary artery segments did not show any injurious effect on endothelium-dependent vascular relaxation, suggesting that this injury is mediated by activated neutrophils. The reduction in neutrophil adherence to the endothelium, and an attenuation of neutrophil-mediated endothelia dysfunction has been strongly associated with reductions in infarct size (Mullane et al., 1984; Forman et al., 1989; Litt et al., 1989). Further experiments, however, are necessary to substantiate the involvement of AMP579 in attenuation of myocardial injury by direct inhibiting neutrophil-mediated events in in vivo model of regional ischemia and reperfusion.

In summary, these data demonstrate that AMP579 is an effective adenosine analog in inhibition of neutrophil activation, adherence and neutrophil-mediated injury to coronary artery endothelium. Direct inhibition of neutrophil-derived superoxide anion generation and degranulation may further amplify the protective effect of AMP579 on neutrophil adherence-dependent dysfunction on vascular endothelium. AMP579, as a new therapeutic agent, may be suitable for attenuating neutrophil-mediated inflammatory reaction at different pathophysiological conditions. However, additional studies are needed to identify possible mechanisms of AMP579 in modulation of adhesion molecules on both neutrophil and vascular endothelium.

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