L-Arginine inhibits neutrophil adherence and coronary artery dysfunction

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Abstract

Background: Nitric oxide (NO) attenuates neutrophil (PMN)-mediated damage, partly by inhibiting superoxide anion (O\textsubscript{2}-) generation and adherence to the coronary artery endothelium. L-Arginine is the endogenous substrate for production of NO via the NO synthase pathway. This study tested the hypothesis that the endogenous NO precursor L-arginine (L-Arg) would reduce PMN-induced coronary artery dysfunction by attenuating O\textsubscript{2}- production and neutrophil adherence. Methods: Neutrophils and left anterior descending (LAD) coronary artery segments were isolated from normal, anesthetised (30 mg/kg i.v. pentobarbitone) dogs. LAD segments were either cut into 2-3 mm rings and mounted in organ chambers to measure vascular tone responses to acetylcholine (endothelium-receptor-dependent) and acidified NaNO\textsubscript{3} (smooth muscle), or cut into segments to measure adherence of fluorescently labeled neutrophils by epifluorescence microscopy. Results: L-Arg had no direct inhibitory effect on O\textsubscript{2}- production (cytochrome c reduction) by PMN activated with platelet activating factor (PAF) (34.6 ± 4.8 nmol vs. 34.2 ± 4.1 nmol). L-Arg (10 mmol) reduced adherence of fluorescently labeled PMN to isolated canine coronary artery endothelium activated by 100 nM PAF from 187 ± 11 to 41 ± 6 PMN/mm\textsuperscript{2}, P < 0.05. This inhibition of adherence was reversed by N-w-nitro-L-arginine (L-NA, 1 mmol) (175 ± 20 PMN/mm\textsuperscript{2}) and by the NO scavenger, carboxy-PTIO (600 μM, 157 ± 23 PMN/mm\textsuperscript{2}). D-arginine, the nonmetabolised enantiomer of L-arginine, (D-Arg, 10 mmol) did not reduce adherence (162 ± 20 PMN/mm\textsuperscript{2}). To determine the effect of PMN on coronary artery endothelial function, canine coronary artery rings were transiently incubated with activated PMNs in organ chambers to induce dysfunction. After washout of PMN, the EC\textsubscript{50} (-log M) derived from post-injury concentration-relaxation responses to acetylcholine was significantly less in 10 mmol L-Arg (6.94 ± 0.08) than untreated rings (6.47 ± 0.06). In contrast, 10 mmol D-Arg could not reverse this dysfunction (6.48 ± 0.11). Conclusions: L-Arg reduces PMN-induced coronary endothelial dysfunction by inhibition of adherence via the L-arginine–NO pathway.

Keywords: Neutrophil activation; Endothelium; Coronary artery tone; Nitric oxide; L-Arginine; L-NAME; Acetylcholine; D-Arginine; Free radicals; PAF; Dog, coronary artery

1. Introduction

Nitric oxide (NO) released by the vascular endothelium possesses many physiologic properties that play a critical role in the maintenance of vascular homeostasis. At physiologic concentrations, NO is a vasodilator [1], as well as an inhibitor of platelet aggregation [2]. NO also possesses potent anti neutrophil activity that includes the inhibition of neutrophil aggregation [3], neutrophil adhesion [4,5] and direct quenching of superoxide anions [6]. Since the anti-neutrophil and anti-oxidant activities of NO released by the coronary endothelium are important cardioprotective mechanisms preventing injury to the endothelium and myocytes, the loss of this endogenous cardioprotection consequent to endothelial damage may be an important component in the pathophysiological progression of myocardial ischaemia–reperfusion injury.

Replacement of NO by an exogenous NO donor, or alternatively augmentation of endogenous NO by supplementation with a precursor of NO (i.e., L-arginine), may...
prevent endothelial damage. It has been shown that exogenous L-arginine increases NO production [7]. L-Arginine is transported into endothelial cells during stimulated release of NO, thereby providing substrate for continued or augmented NO production [8]. In fact, previous studies have demonstrated that supplementation of L-arginine in doses ranging between 3 and 10 mM [9] in both regional ischemic-reperfusion injury models [10,11] and surgical models [12,13] reduces necrosis and preserves endothelial function. In these studies, reduced posts ischemic injury was associated with reduced myeloperoxidase enzyme activity, which is a marker for neutrophil accumulation. These results suggest that the cardioprotective effect of L-arginine, through the L-arginine–NO pathway, may be attributed in part to inhibition of neutrophil adherence to the coronary endothelium, reduction of superoxide production by activated neutrophils [14], and attenuation of neutrophil-dependent endothelial cell damage [5,12]. However, the exact mechanisms of L-arginine cardioprotection observed in vivo have not yet been defined.

We tested the hypothesis that L-arginine, the endogenous physiological precursor of NO, reduces neutrophil adherence to coronary artery, directly decreases the adherence-independent generation of superoxide anions from activated neutrophils, and attenuates neutrophil-dependent coronary artery dysfunction in canine isolated coronary arteries.

2. Methods

The dogs were handled in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication #85-23, revised in 1985). The institutional Animal Care and Use Committee approved the study protocol.

2.1. Surgical procedure

Heartworm-free adult mongrel dogs of either sex weighing 19 to 30 kg (average 22.7 ± 0.7 kg) were initially anesthetised with intravenous sodium pentobarbitone (30 mg/kg). Each dog was endotracheally intubated and ventilated with oxygen-enriched room air using a volume-cycled respirator (Harvard Apparatus, South Natick, MA). The right femoral artery was cannulated to collect peripheral blood for subsequent neutrophil isolation.

The chest was opened by median sternotomy. The pericardium was incised and tented to cradle the heart. Peripheral blood (200 ml) was collected from the femoral artery for isolation of neutrophils. After blood collection, additional sodium pentobarbitone (30 mg/kg) was injected rapidly through the left atrium. The heart was then excised and immediately immersed in cold Krebs-Henseleit (K-H) solution having the following composition (in mmol/l): 118 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 2.5 CaCl₂, 12.5 NaHCO₃, and 10 glucose. The left coronary artery was carefully removed 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 2–3 mm in length.

2.2. Neutrophil isolation

Thirty-milliliter aliquots of peripheral arterial blood were placed in 50 ml conical plastic tubes and mixed with 4.5 ml of anticoagulating agents, which included 1.6% citric acid and 2.5% sodium citrate at pH 5.4, and 10 ml of 6% dextran solution in buffered Hanks balanced salt solution (HBSS). After the erythrocytes had sedimented (approximately 50 min. at room temperature), the leukocyte-rich plasma layer was removed into 50 ml plastic centrifuge tubes and centrifuged at 400 x g for 10 min. at 4°C. Any contaminating red cells in the pellet were removed by hypotonic lysis for 20 s with 9 ml sterile water. Subsequent addition of 3 ml of 0.6 M KCl and a large volume of buffered HBSS rapidly returned these cells to isotonicity. The resulting suspension was centrifuged at 400 x g and the cells were resuspended in 2 ml of HBSS. The cell suspension was then layered on top of 3 ml of Ficoll-Paque (Sigma Chemical, St. Louis, MO) and centrifuged at 600 x g for 20 min again. The pellet was rinsed with Ca²⁺- and Mg²⁺-free HBSS and the cell population adjusted to the desired concentration. Using this procedure, final suspensions contained more than 98% neutrophils and cell viability was greater than 95% as determined by trypan blue exclusion. The isolation process did not activate the PMN, confirmed by absence of shape changes (epifluorescent microscopy), and functional damage to endothelial agonist-stimulated receptors (see Results), lack of contractile response in co-incubation studies with coronary artery segments (see Results), and lack of adherent PMNs (see Results).

2.3. Superoxide production

Superoxide anion (O₂⁻) production by neutrophils isolated from 5 dogs was determined by measuring the superoxide dismutase-inhibitable reduction of ferricytochrome c to ferrocyanochrome c. PMN (5 x 10⁷/ml cells) were prewarmed at 37°C in 160 μmol cytochrome c and 5 μg/ml cytochalasin B, and incubated in L-arginine (1, 5, or 10 mmol) for 5 min if appropriate as treatment group. The PMN were then stimulated with platelet activating factor (PAF, 100 nmol) in a final reaction volume of 0.5 ml. This concentration of PAF was chosen after appropriate dose-response studies for PAF, LTB₄, and FMLP (Table 1). For each assay, duplicate samples were run containing an excess (100 μl) of superoxide dismutase. The reaction was monitored spectrophotometrically by determining the optical density of the supernatant at 550 nm, utilizing a V Max Microtiter Plate Reader (Molecular Devices, Palo Alto, CA).
Effects of platelet activating factor (PAF), leukotriene B4 (LTB4), and n-formyl-Leu-Phe (FLMP) on cytochrome c production and adherence of neutrophils

| Table 1 |
|-----------------|-----------------|
|                | Cytochrome c    | Adherence            |
|                | (nmol O2/5 X 10^7 PMN/ml) | (PMN/mm^2 endothelium) |
| Unstimulated PMN | 6.0 ± 1.1        | 70.3 ± 12.2          |
| PAF             |                  |                    |
| 50 nM           | 10.1 ± 2.2       | 110 ± 2.5           |
| 100 nM          | 31.4 ± 3.9 *     | 262 ± 28.1 *        |
| 1 μM            | 21.3 ± 4.5 *     | 149 ± 10.2 *        |
| LTB4            |                  |                    |
| 50 nM           | 9.2 ± 2.5        | 69.6 ± 10.3         |
| 100 nM          | 7.6 ± 1.9        | 97.5 ± 5.7          |
| 150 nM          | 7.6 ± 1.4        | 97.5 ± 6.1          |
| FLMP            |                  |                    |
| 50 nM           | 4.2 ± 2.3        | 91.1 ± 12.5         |
| 100 nM          | 3.9 ± 1.6        | 93.3 ± 6.8          |
| 150 nM          | 3.9 ± 1.7        | 78.6 ± 6.9          |

* P < 0.05 vs. unstimulated PMN.

CA). Superoxide anion production was calculated using an extinction coefficient of 21.1 nmol⁻¹.cm⁻¹ for cytochrome c. Results are reported as nanomoles of superoxide dismutase-inhibitable O₂⁻ production per 5 X 10⁷ PMN/ml.

2.4. Neutrophil adherence assay

Alterations in the endothelial cell adherence component of neutrophil activity by l-arginine were assessed using neutrophils labeled with Zynaxis PKH-26 vital fluorescent dye (Zynaxis Cell Science, Inc., Malvern, PA). 1.0 ml of diluent and 1.0 ml of PKH2-GL dye (4 μmol) were added to a suspension of isolated neutrophils (approximately 10 million cells) and then mixed for 5 min. 2.0 ml of phosphate-buffered saline (PBS) containing 10% plasma was underlayered in the suspension. Cells were then centrifuged at 600 X g for 10 min at 4°C. The pellet was resuspended in PBS and the number of cells were counted and the labeling procedure yields cells possessing normal viability and function [15]. Autologous PMN suspensions (5 X 10⁶/ml cells) were added into the organ baths alone (PMN, n = 3), PMN + 100 nM PAF (PMN + PAF, n = 10) or in combination with L-Arg (10 mmol, n = 5), L-NA (1 mmol, n = 2) + L-Arg (10 mmol, n = 5) or D-Arg (10 mmol, n = 5). After 10 min, 100 nM PAF was added to the organ chambers and allowed to incubate for 20 min. In one group coronary arteries were not exposed to PMNs and served as undamaged control rings.

The coronary artery rings coincubated with PMNs were washed 3 times with K–H solution to remove PMNs. Dose–response curves to 2.5 to 12.5 nmol/l U46619 (Upjohn Co., Kalamazoo, MI), a thromboxane A₂ mimetic agent, were performed in all rings to determine the optimal concentration of U46619 for precontraction. Once a stable
2.6. Statistical analysis

All data were analyzed with the Statistical Analysis System Program (PC-SAS, SAS Institute, Cary, NC). Time-related differences (incremental drug concentrations) and group–time interactions were analyzed by two-way analysis of variance (ANOVA) for repeated measures adjusted for baseline values followed by Duncan’s Multiple Range post hoc test. Superoxide production and PMN adherence data were compared among the groups using one-way ANOVA followed by Duncan’s post hoc test.

Contraction was obtained, cumulative concentration–response curves to acetylcholine (concentrations from 0.01 to 1.0 μmol/l; Sigma Chemical Co.), a muscarinic receptor-mediated endothelium-dependent stimulator of NO, were obtained. After recording the response to acetylcholine, the rings were washed several times with K-H solution and equilibrated to baseline levels of passive tension (15–20 min). The procedure was repeated for acidified (pH 2.0) sodium nitrite (concentration from 1 to 100 μmol/l; Sigma Chemical Co.), an endothelium-independent smooth muscle relaxing agent which releases NO. Preliminary studies showed no vasodilator effect of acidified buffer without NaNO₂ or non-acidified NaNO₂. All drug concentrations are expressed as the final concentration in the organ chamber.

3. Results

3.1. Superoxide production

Canine neutrophils (5 × 10⁷/ml) incubated with 100 nmol PAF produced 34.2 ± 4.1 nmol/5 × 10⁷/ml PMN superoxide anions. Preincubation with 1 mmol L-Arg (34.1 ± 4.8 nmol), 5 mmol L-Arg (37.3 ± 5.3 nmol), 10 mmol L-Arg (34.6 ± 4.8 nmol) had no direct inhibitory effect on O₂⁻ production by activated PMN (Fig. 1).

3.2. Neutrophil adherence to coronary endothelium

When unstimulated PMN were added to the baths containing normal unstimulated coronary endothelium (PMN alone), very little adherence of PMN (49 ± 6 PMN/mm²) was observed. However, stimulation of the PMN with PAF resulted in a significant increase in the adherence to 187 ± 11 PMN/mm² (P < 0.05 vs. unstimulated). L-Arg incubation inhibited PMN adherence to the endothelium in a dose-dependent manner up to a concentration of 10 mmol (41 ± 6 PMN/mm²) (Fig. 2). The NO-scavenger agent, carboxy-PTIO, also reversed the effects of L-Arg on adherence to 92 ± 23 (300 μM carboxy-PTIO, not shown in Fig. 2) and 157 ± 23 PMN/mm² (600 μM carboxy-PTIO), the latter of which was not significantly different from the PMN + PAF group (Fig. 2). D-Arg (10 mmol), the non-metabolised enantiomer of L-Arg did not inhibit adherence (162 ± 20 PMN/mm²). Attenuation of PMN adherence by 10 mmol L-Arg was totally reversed when the NO synthase blocker L-NA (1 mmol) was added before addition of L-Arg (175 ± 20 PMN/mm²), but adherence was unaltered by 100 μmol L-NA (31 ± 7 PMN/mm², Fig. 3).
L-NA in the absence of 10 mM L-Arg significantly increased adherence, indicating inhibition of basal NO-mediated processes (Fig. 3).

3.3. Coronary artery ring data

3.3.1. PMN-induced contraction

In undamaged control rings not exposed to PMNs, the addition of unstimulated canine PMNs (5 × 10^6/ml cells) to the organ bath did not elicit any contraction. The addition of 100 nmol PAF in the absence of PMN produced no significant contraction of the coronary artery rings (data not shown). However, stimulation of PMN with PAF (100 nmol) resulted in a significant contraction of 0.83 ± 0.13 g above baseline (Fig. 4). L-Arg at a concentration of 10 mmol significantly inhibited the PMN-induced contraction by 78% (0.26 ± 0.10 g, \( P < 0.05 \)) compared to control), while L-NA (1 mM) significantly reversed the effects of L-Arg (10 mmol). L-NA at 100 μM (2.16 ± 0.27 not shown in Fig. 4) and 1 mM in the absence of 10 mM L-Arg significantly increased PMN-mediated contraction. In addition, \( \delta \)-arginine did not inhibit the PMN-induced contraction.

3.3.2. Endothelium-dependent relaxations

Fig. 5(A,B) shows vasodilator responses to acetylcholine in the isolated coronary rings, expressed as a percentage of U46619-induced precontraction. Although acetylcholine caused concentration-dependent relaxation responses in all 6 groups, differences were observed in the magnitude of relaxation among groups. There were no differences in the responses between the control and unactivated PMN groups (Fig. 5A), suggesting that unactivated PMN themselves do not negatively effect the ring responses. However, when activated by 100 nmol PAF (PMN + PAF group), the concentration–response curves in the rings showed significantly decreased responses from the first to the fifth concentrations of acetylcholine (0.01 to 0.685 μmol/l) compared with the curves in the control and PMN groups, suggesting a rightward shift in the concentration–response curve. Accordingly, the \( EC_{50} \) (−log[M]), the concentration of the drug required to effect relaxation to 50% of precontracted levels, also increased in the PMN + PAF (6.75 ± 0.04) group compared with the control (7.61 ± 0.05) and the unstimulated PMN (7.47 ± 0.06) groups, confirming a rightward shift and decreased sensitivity to acetylcholine (Fig. 5C). However, maximum relaxations were not different among the three PAF-stimulated groups (114.3 ± 1.4% for the control group, 113.8 ± 1.9% for the PMN group and 96.9 ± 6.1% for the PMN + PAF group, Fig. 5A). Therefore, PMN-mediated injury was associated with a decreased sensitivity (rightward shift) to acetylcholine without attenuation of the maximum response.

A preliminary study [12] showed that the antagonist effects of L-NA in normal coronary rings persisted in vascular rings despite up to 3 h of continuous washout, manifested as a 70% decrease in the maximal extent of relaxation, a dramatic right shift in the dose–response curve, and a significant increase in the \( EC_{50} \). Therefore, we excluded the L-NA group from in vitro coronary ring data analysis to avoid misinterpretation of the data.

The concentration–response curves in the L-Arg and \( \delta \)-Arg groups were markedly shifted to the right compared
with the curve from the control group (Fig. 5B). However, the responses in the L-Arg group were significantly \( (P < 0.05) \) higher than those in the D-Arg and PMN + PAF groups at the second to the fifth concentrations of acetylcholine \((0.035 \text{ to } 0.685 \mu\text{mol/l})\). The \( EC_{50} \) also increased in the L-Arg \((6.94 \pm 0.08) \) and D-Arg \((6.48 \pm 0.11) \) groups compared with the control \((7.61 \pm 0.05) \) group, but the

increase in the \( EC_{50} \) of the L-arginine treatment group was significantly \( (P < 0.05) \) less compared with the D-Arg and PMN + PAF groups (Fig. 5C).

### 3.3.3. Endothelium-independent smooth-muscle relaxations

Acidified sodium nitrite was used to induce endothelium-independent relaxation of vascular smooth muscle (Fig. 6A). Although sodium nitrite caused concentration-dependent relaxation responses in all 5 groups, differences in the magnitude of relaxation were observed among groups. There were no differences in the responses between the control and PMN groups. However, in the L-Arg and D-Arg groups, the concentration–response curves in the rings showed significantly decreased responses from the second to the fifth concentrations of sodium nitrite \((0.6 \text{ to } 16.6 \mu\text{mol/l})\) compared with the curves in the control and PMN groups. The \( EC_{50} \) also increased in the PMN + PAF \((4.54 \pm 0.05) \), L-Arg \((4.59 \pm 0.07) \) and D-Arg \((4.61 \pm 0.16) \) groups compared with the control \((5.33 \pm 0.05) \) and the PMN \((5.44 \pm 0.07) \) groups (Fig. 6B). However, maximum relaxations were not different among the groups using PAF-activated PMN \((107.8 \pm 2.0\% \) for the control group, \(116.2 \pm 2.1\% \) for the PMN group, \(123.8 \pm 4.4\% \) for the PMN + PAF group and \(120.7 \pm 5.1\% \) for the L-Arg
group) except in the D-Arg group which was significantly greater (143 ± 21.2%) than in the other groups. These results suggest that activated PMNs can cause dysfunction not only to the endothelium but also to the vascular smooth muscle directly. However, there were no group differences among drug-treated groups. Preincubation with L-Arg, but not with D-Arg, preserved the coronary endothelial function but failed to prevent smooth muscle dysfunction from the PAF-activated PMNs.

4. Discussion

It has been demonstrated that supplementation of L-arginine, the endogenous physiological precursor of NO, attenuates ischemia-reperfusion injury in regional coronary occlusion-reperfusion [10,11] and surgical revascularization models [12]. NO is a potent vasodilator [1] as well as a potent inhibitor of platelet aggregation [2]. NO also possesses potent antineutrophil activity that includes the inhibition of neutrophil aggregation [3], neutrophil adhesion [4,5], and direct quenching of superoxide anions [6]. In the present study, L-Arg did not directly inhibit superoxide anions generated by PAF-stimulated PMN in the absence of endothelium (adherence-independent superoxide generation). PAF-activated neutrophils adhered significantly to the coronary artery endothelium, and furthermore produced significant attenuation of both endothelium-dependent and endothelium-independent (vascular smooth muscle) responses to vasodilator agents. L-Arginine inhibited PMN adherence to the endothelium in a dose-dependent manner up to a concentration of 10 mmol, while 10 mM D-arginine had no inhibitory effect. The inhibition of adherence by L-arginine was reversed by 1 mM L-NA, and by an NO scavenger agent, supporting directly the involvement of NO. In addition, L-NA also inhibited PMN adherence in the absence of exogenous L-arginine, suggesting interference with basally released NO. These results suggest that inhibition of PMN adherence by L-arginine involves the L-arginine-NO pathway. L-Arginine inhibited adherence-dependent PMN processes, including PMN-mediated contraction attributed to release of superoxide anions [16], while there was no direct inhibition of adherence-independent superoxide generation. In addition, L-Arg attenuated coronary endothelial dysfunction induced by activated PMN, manifested as significantly greater vasorelaxation responses to ACh. These results suggest that L-Arg has no direct inhibitory effect on adherence-independent superoxide anion production by PMN but prevents neutrophil adherence to the endothelium and subsequent superoxide-mediated vascular dysfunction in our bioassay system.

In the present study, inhibition of neutrophil adherence or dysfunction to vascular rings by L-arginine is dependent on basal release of NO since exogenous stimulators (i.e., acetylcholine, bradykinin) were not used during the co-incubation period. Basal release of NO has been reported previously [9,17] and is suggested by augmented neutrophil adherence by L-NA in the absence of L-arginine in the present study. Although we did not measure the release of NO directly, the inhibitory effects of D-arginine, the NO scavenger carboxy-PTIO, and the NO-synthase inhibitor L-NA strongly suggest that NO was involved in the reduction of neutrophil adherence and endothelial dysfunction. The concentrations of L-arginine used in the present study (1, 5 and 10 mM) were considerably greater than plasma (58 μM) [18] or intracellular (0.4–0.8 mM) concentrations, but in vitro concentrations in the plasma range have shown variable release of NO [7,19] under conditions where the cells are not depleted of L-arginine. Incubation of our cells was relatively short, therefore they were most likely not depleted of L-arginine and were, consequently, relatively less responsive to exogenous administration of the amino acid [20]. The concentrations of L-arginine used in the present study were similar to previous in vivo [10–12] and in vitro [9,10] studies, and exceed the amount of L-arginine necessary to stimulate the normally saturated NO-synthase (EC_{50} = 6 μM) [21]. However, a saturation of NO synthase at this concentration of L-arginine (determined in disrupted cells) [21] is inconsistent with recruited release of NO by greater concentrations of substrate. Higher concentrations of exogenous L-arginine may be required in whole intact cell systems because (1) there may be compartmentalization of either the amino acid or NO synthase thereby shifting the concentration–response curve to the right, (2) they were relatively undepleted and therefore less responsive to exogenous L-arginine, and (3) regulatory mechanisms of NO synthase by calcium and co-factors may differ from cell-free systems. Concentration–response curves in Fig. 2 suggest that concentrations of L-arginine lower than 1 mM may not be effective in inhibiting neutrophil adherence in our preparation.

To determine whether the anti-neutrophil properties of L-arginine required the interaction between PMN and endothelial cells, the adherence of PAF-stimulated PMN to normal coronary artery endothelium was measured. The initial prerequisite step in PMN activation and the subsequent steps in the inflammatory cascade leading to cellular injury in ischemic-reperfused myocardium involves interaction with and adherence to coronary artery and venous endothelial cells [22,23]. Once the PMN has adhered to the vascular endothelium, a series of events involving "rolling" and firm attachment to the endothelial surface, diapedesis, and later extravascular emigration into neighboring cardiac myocytes can occur [23]. Studies suggest that these events occur primarily after reperfusion [24]. Interference by L-arginine, via the NO pathway, with adherence-dependent mechanisms rather than adherence-independent mechanisms is suggested by (1) reduced adherence to the endothelium, (2) inhibition of PMN-mediated constriction by eliminating NO by a quenching reaction with superoxide anions [16], and (3) the failure of L-arginine
to inhibit adherence-independent superoxide anion production. In the present study, L-arginine preincubation inhibited the PAF-stimulated PMN adherence to the endothelium in a dose-dependent manner up to a concentration of 10 mmol. The maximum inhibition of PMN adherence by 10 mmol L-arginine is consistent with the results of our previous studies [11,12] which show that L-arginine at or below this dose is cardioprotective by inhibition of PMN accumulation in the myocardium as suggested by reduced tissue myeloperoxidase activity. The involvement of NO in L-arginine's effects, rather than another mechanism, is demonstrated by reversal of the precursor's inhibitory effect by an NO scavenger carboxy-PTIO.

In vitro studies, we examined the effect of L-arginine on neutrophil-induced coronary artery endothelial contraction under basal conditions purportedly produced by neutrophil-generated superoxide anions [16]. This oxygen free-radical production by PMN degrades NO in a biradical coupling reaction [6] which overwhelms the basal release of NO by the coronary vascular endothelium and, hence, withdraws associated basal vasodilator tone resulting in contraction of the coronary rings [9]. The observation in the present study that incubation of PMN and arterial rings with L-arginine was associated with greater contractions is consistent with a basal release of NO [9]. Previous studies using isolated coronary arterial preparations and autologous PMN from cats [16], dogs [5] and humans [25] have demonstrated profound vasoconstriction upon activation of the PMN by a number of stimuli, including low-flow perfusion followed by a return to normal perfusion [16]. Furthermore, these PMN-induced coronary artery contractions have been shown to be associated with endothelial dysfunction characterised by attenuated responses to endothelium-dependent vasodilators [16] and is comparable to the PMN-mediated coronary endothelial dysfunction that occurs following myocardial ischemia-reperfusion [26]. In the present study, L-arginine significantly inhibited the PMN-induced contraction of coronary artery rings and provided a significant degree of endothelial protection as assessed by the vasorelaxation responses to ACh most likely by interfering with adherence. In contrast, L-arginine could neither inhibit the PMN-induced contraction nor prevent endothelial dysfunction, while L-NA aggravated PMN-induced contraction. These results provide additional evidence for inhibition of neutrophil activities by mechanisms involving the L-arginine–NO pathway.

In this study, we used PAF as a stimulator of neutrophils. In preliminary studies, alternative stimulators such as FMLP and LTβ were found to be less potent stimulators of superoxide production by PMN. In our study, we used 100 nmol PAF which is sufficient to induce PMN-dependent interaction with the coronary vascular endothelium as supported by our adherence data. In contrast to other studies from our laboratory [5,11,12,27,28] and others [10,29] using models of ischemia-reperfusion injury, smooth muscle dysfunction was observed as well as endothelial dysfunction. PAF causes PMN to degranulate, thereby releasing proteases including elastase [30], as well as the enzyme, myeloperoxidase [31]. These proteolytic enzymes are responsible for breakdown of the collagenous, elastic and proteinaceous components of the extracellular matrix. Therefore, PAF-stimulated PMN can cause not only endothelial damage but also vascular smooth muscle damage leading to attenuated agonist-stimulated relaxation responses. This may explain not only the observed smooth muscle by dysfunction, but also the incomplete recovery of endothelial function in the L-arginine-treated coronary artery rings in the present study. These results clearly show that L-arginine can protect against adherence-dependent endothelial damage but not smooth muscle damage. In our study, we did not use a proteinase inhibitor. However, there were no group differences in segments co-incubated with PAF-activated PMN that might confound interpretation of the data.

Whether NO under physiological conditions and pathophysiological conditions is cytotoxic or cytoprotective is highly controversial. The actions of L-arginine and presumably stimulated NO production may be concentration-dependent as well as model-dependent. Low concentrations of NO produced by endogenous or low-dose L-arginine or supplementation with NO-donor agents may be beneficial in the setting of in vivo ischemia-reperfusion [10,11,32–34], while higher concentrations generated in vitro by exogenous authentic NO or NO-donors may produce unphysiological concentrations of NO. Excess concentrations of NO may favor the production of peroxynitrite by biradical coupling between NO and superoxide anions, with subsequent peroxynitrite protonation to produce hydroxyl radicals and nitrogen dioxide [35,36]. Both peroxynitrite and hydroxyl radicals are cytotoxic, and the presence of peroxynitrite in vitro has been observed to produce tissue damage in some [35] but not all studies [36]. Whether peroxynitrite and hydroxyl radicals are produced from NO in vivo is unclear and arguable [37]. There is evidence to suggest that peroxynitrite is “recycled” to NO in plasma environments, or that NO may be “trapped” as an adduct of plasma albumin which would kinetically favor less peroxynitrite production [37]. However, in the present in vitro study in which endogenous NO production is certainly less than administration of exogenous NO sources, protective effects were observed.

In summary, the present study demonstrates that L-arginine, the physiological precursor of NO, inhibits PAF-stimulated PMN adherence to endothelium and protects against endothelium-dependent but not smooth muscle damage. These results confirm conclusions made in previous studies that L-arginine is cardioprotective [10–12] in models of myocardial ischemia and reperfusion by inhibition of neutrophil accumulation. Additional studies utilizing NO-donor agents are necessary to determine if exogenous NO can work in the same manner as endogenous NO in reducing PMN-mediated coronary vascular injury.
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