Release of a neutrophil-derived vasoconstrictor agent which augments platelet-induced contractions of blood vessels *in vitro*

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1 The effects of neutrophil-derived products on vascular tone *in vitro* were examined by adding purified rabbit neutrophils to siliconized organ baths containing rings of rabbit thoracic aorta.

2 Neutrophil-derived products induced a concentration-dependent contraction of the blood vessels generating 3.9 ± 0.2 g of tension at a cell concentration of 2×10^6 ml⁻¹. This contractile response was not dependent on an intact endothelium and was not ameliorated by treatment of the neutrophils with inhibitors of lipoxygenase or cyclo-oxygenase enzymes, by free radical scavengers or by the use of end organ antagonists to angiotensin II, desArg⁹-bradykinin, histamine, catecholamines or acetylcholine.

3 Neutrophils stimulated with phorbol myristate acetate, formyl methionyl-leucyl-phenylalanine or calcium ionophore A23187 released a contractile factor into the supernatant which produced qualitatively similar contractions compared to those elicited by incubation with intact unstimulated neutrophils.

4 Ten to twenty times more platelets were required to evoke equivalent contractions to those observed with neutrophils. However, neutrophil supernates significantly augmented platelet-mediated contractions ($P < 0.001$).

5 Contractions elicited by neutrophils and supernates derived from activated neutrophils were partially antagonized by 5-hydroxytryptamine (5-HT) receptor antagonists, methysergide and ketanserin. However, the inhibition of exogenous 5-HT-induced contractions on rabbit aorta and rat stomach strips by both antagonists was greater than the inhibition of contractions produced by neutrophils and neutrophil-derived supernates.

6 Extraction of the biologically active material from supernatants of activated neutrophils into acetone, but not chloroform-methanol or ethyl acetate, suggests the contractile factor may be a protein/peptide. Partial purification on a Sephadex G100 column yields a contractile factor with a molecular weight of less than 4,000 daltons.

7 This factor may augment vascular tone, either directly or by interactions with platelets, in pathophysiological states associated with neutrophil activation and accumulation.

Introduction

Neutrophils adhere to the vascular endothelium and accumulate at discrete sites within the circulation in many pathological states including acute inflammatory disorders, myocardial infarction, respiratory distress syndrome, hypertension and endotoxin or haemorrhagic shock (Brigham & Meyrick, 1984; Chobanian *et al.*, 1984; Schmid-Schonbein & Engler, 1986; Mullane, 1988). The interaction of neutrophils and blood vessels may be important in the regulation of vascular function since activated neutrophils release a variety of factors that are capable of influencing vascular tone.

The range of neutrophil-derived factors possessing vasoactive properties include reactive oxygen species, lipid metabolites and proteolytic enzymes. Activated neutrophils generate oxygen-derived metabolites, superoxide anion and hydrogen peroxide (H₂O₂) (Babior, 1984) which may indirectly affect vascular tone by inactivating endothelial-derived relaxing factor (EDRF) (Gryglewski *et al.*, 1986) and by diminishing prostacyclin release (Whorton *et al.*, 1985), respectively. Conversely, H₂O₂ itself may evoke vascular relaxation due to stimulation of guanylate cyclase (Burke & Wolin, 1987). Lipid-derived mediators, platelet-activating factor and 15-hydroperoxyicosatetraenoic acid provoke endothelial-dependent relaxations of isolated blood vessels (Kamitani *et al.*, 1984; Thomas & Ramwell, 1986). Cathepsin G, a proteolytic enzyme released from neutrophil azurophilic granules, cleaves angiotensinogen to release angiotensin II, which con-

tracts vascular smooth muscle (Wintroub *et al.*, 1984). However, it is not clear whether such factors are released from neutrophils interacting with blood vessels. In addition, some neutrophil-derived mediators including platelet activating factor (PAF), hypochlorous acid, cathepsin G and elastase can augment platelet activation and the release of mediators from platelets (see Mullane 1989, for review). The addition of platelets to organ baths containing rings of blood vessels provokes a contraction, attributed to 5-hydroxytryptamine (5-HT) and thromboxane A₂ (TxA₂; Cohen *et al.*, 1983; Houston *et al.*, 1985; 1986).

The present study was designed to investigate the functional significance of neutrophil/blood vessel interactions and to elucidate the role of various classes of neutrophil-derived factors on vascular tone. We show that neutrophils elicit a concentration-dependent contraction of isolated rabbit aortic rings, which apparently is not mediated by oxygen radicals, arachidonic acid metabolites, catecholamines, acetylcholine, angiotensin II or desArg⁹-bradykinin. We also demonstrate that activated neutrophils release a factor into the supernate which contracts vascular rings *in vitro* and potentiates platelet-induced contractions. A thermostable, low molecular weight material of non-lipidic nature which elicits a contractile response in rabbit aortic rings is preliminarily identified.

Methods

Male New Zealand white rabbits (3–3.5 kg) were anaesthetized by an intramuscular injection of ketamine HCl (Ketaset, 50 mg kg⁻¹) and xylazine (Rompum, 8 mg kg⁻¹).

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Assay of vascular smooth muscle contractile activity

The rabbit thoracic aorta was gently removed and placed into ice cold Krebs-bicarbonate buffer (pH 7.4) and cut into 2 mm wide rings. The composition of the Krebs-bicarbonate solution was (g l^{-1}): NaCl 6.92, KCl 0.354, CaCl_2 0.280, KH_2PO_4 0.162, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.294, NaHCO_3 2.1 and dextrose 2.0. Aortic rings were mounted in 5 ml silicized water-jacketed organ baths maintained at 37°C and continuously gassed with 95% O_2 :5% CO_2 . Tissues were equilibrated under 2 g of resting tension for 2 h. The solution in the organ baths was changed at 15 min intervals and basal tension was adjusted as required during the equilibration period. Two grams of basal tension was optimal for concentration-response curves to both phenylephrine and cell extracts. Changes in tension were measured with Grass model FTO3C force transducers coupled to a Hewlett-Packard 775BA chart recorder.

Isotonic contractions of rat fundic strips were used as a bio-assay for 5-HT-mediated contractions (Vane, 1957). The fundic portion of the stomach was removed, cut into a strip 4 to 5 cm long, a thread attached to each end, and mounted with a 1.5 g preload in a water-jacketed organ bath maintained at 37°C . The strip was superfused with Krebs-bicarbonate buffer (pH 7.5) containing indomethacin ($1 \mu\text{g ml}^{-1}$) and scopolamine ($1 \mu\text{g ml}^{-1}$) at a flow rate of 4 ml min^{-1} . Contractions were monitored via Harvard transducer coupled to a Soltec 1244 recorder.

Preparation of rabbit neutrophils

Anaesthetized rabbits were exsanguinated via a cannula inserted into the abdominal aorta. Blood was collected into two 50 ml syringes each containing 5 ml of sodium citrate (3.15, w/v). The citrated blood was mixed (1.9 v/v) with 6% dextran (mol. wt. 500,000) and erythrocytes were allowed to sediment for 2 h at room temperature. The supernate was centrifuged (180 g) for 5 min and the cell pellet subjected to hypotonic lysis of residual erythrocytes. Leukocytes were washed with 0.9% saline, layered onto a ficoll-hypaque density gradient (Histopaque 1077, Sigma) and centrifuged at 200 g for 20 min. The neutrophil-rich pellet in which 90 to 95% of the cells were neutrophils, was suspended in Hank's buffered saline at a concentration of 1×10^7 to 2×10^7 cells ml^{-1} . Cell suspensions contained $1.75 \pm 0.56 \times 10^7$ neutrophils ml^{-1} with a platelet contamination of $2.5 \pm 0.45 \times 10^7$ platelets ml^{-1} ($n = 4$). Examination of the cell suspension by light microscopy revealed greater than 99% viability as assessed by Trypan blue exclusion.

Preparation of neutrophil-derived contractile material

Aliquots of neutrophil suspensions (10^6 – 10^7 cells) were placed in conical Eppendorf 1 ml microfuge tubes and incubated with phorbol myristate acetate (PMA, 0.016 and $1.6 \mu\text{M}$), formyl methionyl-leucyl-phenylalanine (FMLP, $1 \mu\text{M}$) or calcium ionophore A23187 ($1.9 \mu\text{M}$) for 15 min at room temperature followed by centrifugation for 60 s in an Eppendorf microfuge. Cell supernates were then added to organ baths and changes in tension measured. In other experiments, 200 μl supernate prepared from 10^7 neutrophils stimulated with A23187 was added to the aortic rings before the addition of platelets (5×10^5 – $1 \times 10^8 \text{ ml}^{-1}$). Protein concentration of cell supernates was measured by the method of Bradford (1976), with bovine serum albumin used as a standard. For purification of the contractile factor, supernates from agonist-stimulated neutrophils (5×10^7 – 8×10^7) were slowly added to ice cold acetone (1:10 v/v) and stirred for 1 h. Acetone precipitable proteins were filtered, washed with ether, dried under N_2 and reconstituted in Tris buffer (0.1 M, pH 7.4).

The crude neutrophil extract was then applied to a Sephadex G-100 gel filtration column for estimation of molecular weight. The column was equilibrated with 50 mM Tris-HCl/200 mM NaCl buffer (pH 7.4 at 4°C at a flow rate at

1 ml min^{-1}). Fractions were collected every 30 s, and the protein concentration monitored spectrophotometrically at 280 nm u.v. Blue dextran was used for determining the void volume of the column and the following protein standards (2 mg each) were used for calibration of molecular weight: bovine serum albumin (mol. wt. 67,000), chymotrypsin (25,000) and ribonuclease A (13,700). At each step of the isolation (acetonic extract, G-100 eluate) activity was verified by bio-assay on rabbit aortic rings.

Preparation of washed rabbit platelets

Citrated whole blood was centrifuged at 1000 g for 15 min to obtain platelet-rich plasma. The plasma was then subjected to hypotonic lysis to remove residual erythrocytes. The erythrocyte-free plasma was centrifuged at 1500 g for 1 h, resultant pellets combined, resuspended in 50 ml saline-EDTA, pH 7.4 and centrifuged at 5000 g for 10 min. The pellet was then re-centrifuged at 200 g for 5 min. The low-speed supernate, free of contaminating elements, was then centrifuged at 4000 g for 20 min to obtain a pure platelet pellet. The platelet pellet was re-suspended in saline (pH 7.4) at a final concentration of 5×10^8 – 8×10^8 platelets ml^{-1} ($n = 8$), which were free of leukocyte contamination.

Experimental protocol

Cumulative concentration-response curves to phenylephrine (10^{-9} – 10^{-6} M) were obtained before the addition of neutrophils to assess individual tissue responsiveness. Accordingly, after each experiment, phenylephrine concentration-response curves were repeated and results expressed as a percentage of the maximal phenylephrine response. The maximum tension developed at 10^{-6} M phenylephrine was $5.24 \pm 0.12 \text{ g}$ ($n = 20$).

After initial phenylephrine concentration-response curves, tissues were washed and neutrophils (1×10^5 – $2 \times 10^6 \text{ ml}^{-1}$) or platelets (5×10^5 – $1 \times 10^8 \text{ ml}^{-1}$) were added in a cumulative fashion to the organ baths. Increasing amounts of blood elements were added only after the maximal response was obtained to the preceding concentration.

In studies with receptor antagonists of various vasoactive mediators, tissues were pretreated 15 min before neutrophil addition. The antagonists and the concentrations (μM) used were as follows: saralasin (1.0), $^8\text{Leu-desArg-bradykinin}$ (1.0), pyrilamine (0.3), propranolol (2.0), phenoxybenzamine (0.4), scopolamine (0.4), methysergide (0.1) and ketanserin (0.1). In some experiments, neutrophils and/or tissues were pretreated with the cyclo-oxygenase inhibitor, indomethacin ($3 \mu\text{M}$) or lipoxygenase inhibitors, BW755C (3-amino-1-[m -(trifluoromethyl)-phenyl]-2 pyrazoline, $50 \mu\text{M}$) or REV-5901 (1-pentyl-3-(2-quinolinylmethoxy)benzene methanol $5 \mu\text{M}$), or with scavengers of oxygen derived free radicals, superoxide dismutase and catalase (both at 60 U ml^{-1}).

In experiments assaying the contractile activity of material released from activated neutrophils, supernates from vehicle-treated cells and the direct effects of the neutrophil stimulating agents (PMA, calcium ionophore and FMLP prepared in Hanks buffered saline) served as controls.

Statistical analysis

Results are expressed as mean $\pm$ s.e. The data were analyzed by a two-way analysis of variance. If statistical differences were noted, multiple comparisons were analyzed by a Bonferroni test. EC_{50} values and maximal responses were evaluated by Student's t test for unpaired data.

Results

Neutrophil-induced contractions of rabbit aorta

The cumulative addition of neutrophils (1×10^5 – 2×10^6 cells ml^{-1}) to the organ baths elicited concentration-dependent iso-

metric contractions of rabbit thoracic aortic rings (Figure 1a). The initiation of the contractile response occurred within 60 s after the addition of neutrophils and was sustained while the neutrophils were present in the organ bath (>30 min). Upon washout, tension declined back to baseline values. Figure 1b depicts the absolute changes in tone, expressed in g. The same data, expressed as percentage of maximal contraction to a standard agonist (phenylephrine $1 \mu\text{M}$) permitted comparisons of neutrophil-derived contractions to those obtained for another vasoactive agent (Figure 1c). The addition of 2×10^6 cells ml^{-1} achieved 80% of the maximal response to PE. The subsequent addition of neutrophil stimulants, FMLP or A23187, did not increase further the neutrophil-mediated responses.

Contamination of neutrophil preparations with platelets

Ficoll-hypaque prepared neutrophils were contaminated with platelets, where platelet:neutrophil ratios varied between 1.4:1 to 2.2:1. Because activated platelets release the vasoconstrictor mediators TxA_2 and 5-HT and the addition of platelet suspensions to organ baths containing rings of canine coronary arteries elicit a contraction (Cohen *et al.*, 1983; Houston *et al.*, 1985; 1986) it was possible that the contractions observed with the neutrophil preparations could be attributed to the contaminating platelets. The cumulative addition of platelets (5×10^5 – $1 \times 10^8 \text{ ml}^{-1}$) to the suspended aortic rings evoked a concentration-dependent contraction (Figure 2). The EC_{50} was $8 \pm 0.9 \times 10^6$ platelets ml^{-1} which was ~ 10 fold higher than the concentration of neutrophils required to elicit a similar response ($P < 0.01$, ANOVA), and ~ 8 fold higher than the number of platelets contaminating the neutrophil suspensions. Moreover, the magnitude and time-course of neutrophil-induced contractions were not influenced by the presence or absence of an intact endothelium, in contrast to the responses observed upon adding platelets (Cohen *et al.*, 1983; Houston *et al.*, 1986). For example, when neutrophils were added to the organ baths containing endothelial intact and denuded vessels, no significant differences in tone were observed (0.3 ± 0.06 vs. 0.5 ± 0.15 , 1.0 ± 0.13 vs. 1.3 ± 0.21 , 2.4 ± 0.58 vs. 2.4 ± 0.53 g of tension developed in intact and denuded vessels, respectively at 10^5 , 2×10^5 and 10^6 neutrophils ml^{-1} added to the organ baths, $n = 4$). Finally, pretreatment of rabbit neutrophils (5×10^6) with

Figure 2 Platelet-induced concentration-dependent contractions of rabbit thoracic aortic rings in the absence (▲) and in the presence (Δ) of neutrophil supernate ($200 \mu\text{l}$). For comparison, the concentration-response curve to neutrophils (●) is included ($n = 36$). Each data point represents tension development in response to the platelet suspension alone ($n = 30$) or in the presence of neutrophil supernate ($n = 16$).

indomethacin ($3 \mu\text{M}$ for 15 min) did not impair neutrophil-mediated contractions, yielding 113% of the control response compared to vehicle-treated cells run in parallel incubates ($n = 2$). This observation argues against a role for TxA_2 (or other vasoconstrictor prostanoids) in the contractile response.

Treatment of neutrophils with inhibitors of arachidonic acid metabolism and free radical scavengers

Pretreatment of rabbit neutrophils (5×10^6) for 15 min with inhibitors of arachidonic acid metabolism, BW 755C ($50 \mu\text{M}$) or REV-5901 ($5 \mu\text{M}$), did not diminish contractions provoked by 5×10^6 cells, with 100 and 103% of the control response being obtained, respectively ($n = 2$). Furthermore, pretreatment of cells (5×10^6) with scavengers of O_2 -derived mediators, superoxide dismutase and catalase (60 u ml^{-1} of each) did not attenuate the neutrophil-induced contractions with treated cells producing 102% of the control response (mean values from two experiments).

Treatment of blood vessels with pharmacological antagonists

Incubation of vessels for 15 min with an antagonist mixture consisting of pyrilamine, propranolol, phenoxybenzamine and scopolamine blocked the contractile response induced by the appropriate agonists added at a concentration of $0.5 \mu\text{M}$. However, the antagonists did not attenuate the contractile response to neutrophils added to the organ baths (Figure 3). In addition, treatment of vessels with saralasin and $^8\text{Leu-desArg-bradykinin}$ did not impair neutrophil-mediated contractions (i.e. 10^6 cells ml^{-1} added to the organ baths generated 1.2, 1.2 and 1.3 g of tension for control-, saralasin- and $^8\text{Leu-desArg-bradykinin}$ -treated vessels, respectively) whereas the responses to angiotensin II and $\text{desArg}^9\text{-bradykinin}$ (both at $1 \mu\text{M}$) were completely abolished. Indomethacin or superoxide dismutase and catalase treatment of aortic rings also did not reduce the cell mediated constrictor responses to 5×10^6 cells ml^{-1} with 4.7, 4.7 and 4.4 g of tension generated for control, indomethacin or superoxide dismutase and catalase-treated vessels (mean values from two experiments each).

In contrast, the 5-HT antagonist, methysergide, attenuated the contractions induced by neutrophils, displacing the cell concentration-response curve to the right with an increase in the EC_{50} values from $5.2 \pm 0.19 \times 10^5$ to $1.04 \pm 0.15 \times 10^6$ neutrophils ml^{-1} ($P < 0.025$, $n = 6$, Figure 4). Similar results were obtained with the 5-HT₂-receptor selective antagonist, ketanserin, which increased the EC_{50} value to $1.01 \pm 0.07 \times 10^6$ cells ml^{-1} ($P < 0.005$, $n = 6$). This represents displacement of the cell concentration-response curves

Figure 1 Neutrophil-induced concentration-dependent contractions of rabbit thoracic aortic rings: (a) depicts a typical trace of a cumulative concentration-response curve performed by the addition of purified rabbit neutrophils to organ baths containing arterial rings. The data generated are then expressed as (b) g of tension, or (c) percentage of maximal response to phenylephrine (10^{-6} M , $n = 20$).

Figure 3 Lack of effect of pyrilamine, phenoxybenzamine, propranolol and scopolamine (○) on neutrophil-mediated contractions of rabbit aortic rings (●, control). The antagonist mix was added to the organ baths 15 min prior to the cumulative addition of neutrophil-enhanced suspensions.

of 2 and 1.9 fold to the right with methysergide and ketanserin, respectively. These results suggest that a portion of the vasoconstrictor activity can be attributed to 5-HT.

Biological activity of supernates from activated neutrophils

To assess if neutrophils can release a mediator that induces contraction of rabbit aortic rings, neutrophils were activated by incubation with an agonist for 15 min, the incubate rapidly centrifuged (1000g, 1 min) and the cell supernates added to the organ baths. Treatment of neutrophils (5×10^6) with three different agonists; PMA (0.016 and 1.6 μ M), calcium ionophore A 23187 (1.9 μ M) or FMLP (1 μ M) provoked the release of a vasoactive substance into supernates, which was not found in vehicle-treated neutrophil supernates (Table 1). Moreover, the direct addition of the neutrophil agonists themselves to the organ baths did not alter vascular tone. In addition, FMLP (1 μ M) treatment of neutrophils (5×10^6) evoked the time-dependent release of vasoconstrictor activity into the supernatant (0.8, 1.3 and 2.3 g of tension at 2, 10 and 30 min incubations of neutrophils, $n = 2$). The contractions of the rabbit aorta produced by the cell supernates were not attenuated by the antagonist mixture, but were partially antagonized by ketanserin (Figure 5). This antagonism was characterized by a reduction in the maximal contraction with no change in the EC₅₀ value. In contrast, the contractions provoked by 5-HT (5×10^{-7} to 7.5×10^{-6} M) were displaced to the right in parallel by ketanserin and methysergide, with significant

Figure 4 Neutrophil-induced contractions of rabbit thoracic aortic rings in the absence (●) and the presence of ketanserin (□) or methysergide (○). Both 5-hydroxytryptamine receptor antagonists significantly reduced neutrophil-mediated contractions at all concentrations of cells added (ANOVA). * $P < 0.05$ for EC₅₀ values (unpaired t test).

Table 1 Activation of neutrophils (5×10^6) by different agonists evokes the release of a vasoactive material into the supernate

Agonist	T (g)	% phenylephrine response
Cells (5×10^6) alone (10)	3.3 ± 0.2	57 ± 7.0
Control supernate (5)	0.1 ± 0.1	2 ± 0.5
Phorbol myristate acetate		
0.016 μ M (3)	0.4 ± 0.1	13 ± 6.0
1.6 μ M (6)	1.6 ± 0.2	25 ± 2.8
Calcium ionophore		
1.9 μ M (4)	0.9 ± 0.4	20 ± 3.0
Formyl methionyl-leucyl-phenylalanine 1 μ M (4)	0.8 ± 0.2	22 ± 2.0

Values represent mean $\pm$ s.e., the number of observations in parentheses.

increases in the EC₅₀ values, without diminutions in the maximal response (Figure 6). Moreover, a comparison of equi-constrictor concentrations of 5-HT (3×10^{-6} M) and the neutrophil-derived material (100 μ l) were compared in the absence and presence of ketanserin (10^{-7} M). 5-HT produced 2.1 ± 0.3 g of tension which was reduced 93% by ketanserin to

Figure 5 Aortic contractions elicited by supernates from activated neutrophils in the absence (●) and presence of pyrilamine, phenoxybenzamine, propranolol and scopolamine (○) or ketanserin (□). PMNs (1×10^6 to 6×10^6) were incubated with calcium ionophore A23187 (1.9 μ M) for 15 min at room temperature, rapidly centrifuged and supernates added to the organ baths in a cumulative fashion. * $P < 0.05$.

Figure 6 Cumulative dose-response curves to 5-hydroxytryptamine (5-HT) in rabbit aortic rings in the absence (●) and presence of ketanserin (□) or methysergide (○). In the presence of both receptor antagonists, significant differences were noted at all concentrations of 5-HT added. * $P < 0.05$.

Table 2 Comparison of extraction procedures used to isolate the contractile material from neutrophil supernates

	T (g)	% phenylephrine response
Ethyl acetate – organic	0	0
– polar	2.0	54
Chloroform : methanol – organic	0.5	7
– polar	1.5	22
Acetone ppt	3.8	61

0.14 ± 0.06 g tension ($n = 7$). In contrast, the neutrophil supernate elicited 2.13 ± 0.31 g of tension by itself, which declined 33% to 1.43 ± 0.37 g of tension with ketanserin ($n = 8$). Similar results were obtained on the rat stomach strip treated with methysergide where the dose ratios for 5-HT and the neutrophil-derived material (the concentrations of agonists required to induce a response of similar magnitude) in the presence and absence of methysergide were approximately 5,000 for 5-HT and 300 for the neutrophil factor. These results imply that a portion of the contractions elicited by cell supernates can also be attributed to 5-HT, as was observed with the neutrophils themselves.

Neutrophil supernates augment platelet-induced contractions

To determine if neutrophil supernates influenced the contractions provoked by washed platelets, 200 μ l of supernate prepared from 10^7 neutrophils stimulated with A23187 (1.9μ M) was added to 2 ml organ baths containing aortic rings, followed by the cumulative addition of platelets. The supernate alone increased vascular tone by 0.24 ± 0.05 g ($n = 16$) and potentiated platelet-induced contractions. The concentration of platelets required to elicit 50% maximum response (EC_{50}) was decreased from $8.0 \pm 0.9 \times 10^6$ ml $^{-1}$ to $2.8 \pm 0.7 \times 10^6$ ml $^{-1}$ by the neutrophil supernate ($P < 0.001$; $n = 16$; Figure 2). This EC_{50} value is close to that of neutrophils alone ($1.0 \pm 0.35 \times 10^6$ ml $^{-1}$, $n = 36$), although the difference is still statistically significant ($P < 0.05$). Other volumes of cell super-

nate were not examined. In contrast the addition of A23187 (0.2μ M) alone did not influence the contractile activity of added platelets.

Extraction, purification and bioassay of the neutrophil-derived contractile factor

The supernates of neutrophils (1×10^7 to 3×10^7) activated with PMA (0.016μ M) or A23187 (1.9μ M) were first subjected to lipid extraction with ethyl acetate or chloroform:methanol (2:1). Minimal biological activity was observed in the organic phase with most of the activity remaining in the polar phase (Table 2). Conversely, supernatant proteins from activated neutrophils precipitated with cold acetone and reconstituted in Tris buffer (0.1 M, pH 7.4), contained a factor which enhanced vascular tone, suggesting that the mediator is protein-like or is bound to a protein. Boiling of the acetone extract from the supernatants of 5×10^7 PMA-activated neutrophils for 15 min resulted in a 22% reduction in biological activity.

Subsequent Sephadex G-100 purification of 2 mg of semi-purified protein (after acetone extraction) yielded 2 peaks of u.v. absorbance at 280 nm (Figure 7). The first peak coelutes with the void volume and is devoid of vasoconstrictor activity. The second peak elutes after the low molecular weight standard, and contains all the vasoconstrictor activity. Consequently, the molecular weight is approximated at less than 4 kD.

Discussion

The present study demonstrates that the addition of rabbit neutrophils to organ baths containing rings of rabbit thoracic aorta provokes a concentration-dependent contraction. The increase in vascular tone is apparently not secondary to neutrophil-mediated tissue damage because rinsing the organ baths with fresh buffer returns tone to basal levels, and concentration-response curves established to phenylephrine before and after the addition of neutrophils do not differ significantly. The neutrophil-mediated contraction of vascular rings is not mediated by eicosanoids or oxygen metabolites derived from neutrophils because neither inhibitors of cyclo-oxygenase and lipoxygenase enzymes, nor superoxide dismutase and catalase, attenuate the vasoconstriction. Moreover, receptor antagonists to acetylcholine, catecholamines, histamine, angiotensin II and des-Arg⁹-kinins are without effect. These observations indicate that the vasoconstriction cannot be attributed to products such as 15-HPETE or angiotensin II formed by neutrophils (Thomas & Ramwell, 1986; Wintroub *et al.*, 1984).

The preparation of neutrophils by ficoll-hypaque procedures results in significant platelet contamination. It is well recognized that platelets can provoke vasoconstriction by the formation of TxA₂ and the liberation of 5-HT (Ellis *et al.*, 1976; DeClerck & David, 1981). Consequently, the possibility that the vasoconstriction attributed to the neutrophils was in reality due to contaminating platelets was examined. However, there are a number of inconsistencies with this proposal. First the response was concentration-dependent to neutrophils and independent of the number of contaminating platelets. The number of platelets required to elicit contractions of a similar magnitude were 8 to 10 fold higher than those found in the neutrophil preparations. Moreover, endothelial denudation of the aortic rings did not modify the contractions to the cell suspensions, in contrast to the platelet-mediated response (Houston *et al.*, 1986). Stimulation of neutrophils by FMLP, which has no effect on platelets (Issekutz *et al.*, 1983), evokes the release of vasoconstrictor material. Taken together, these observations do not support a role for platelets as the principal source of mediators of the aortic contractions.

Figure 7 Partial purification of the neutrophil-derived vasoconstrictor material by Sephadex G-100 chromatography. Neutrophil supernates were extracted with cold acetone (1:10) and applied (2 mg protein) to the G-100 column. Peaks of u.v. absorbance from neutrophil supernates (●), molecular weight standards (○). Hatched columns represent column fractions assayed for vasoconstrictor activity in rabbit aortic rings. The biologically active material was eluted between fractions 120 and 140.

It can be argued that the addition of neutrophils to silicized organ baths enables the cells to interact with components of the blood vessel or surfaces (silica, metal rings) which they may not encounter *in vivo*. However, useful insights into mechanisms by which platelets affect vascular tone have been provided by similar experimental procedures (Cohen *et al.*, 1983; Houston *et al.*, 1985; 1986). Moreover, this approach permits the observation of responses to labile substances as well as synergistic interactions between different mediators, which may not be reproduced by the exogenous addition of an individual mediator. We assume that the neutrophils are being activated upon addition to the organ baths because the subsequent addition of a stimulus such as FMLP does not augment the response. To investigate further whether neutrophil activation is required for the vascular contractions, the cells were stimulated *in vitro* and the supernates added to the baths. Irrespective of the stimulus used (PMA, calcium ionophore or FMLP), the neutrophil supernates evoked contractions of the aortic rings. However, it should be noted that the contraction elicited by the cell supernates rarely exceeded 50% of that induced by addition of the cells themselves. This may imply that either the physical presence of the neutrophil is required for full expression of the response via direct cell-cell interactions, or that a labile mediator also contributes to the contraction.

Contractions induced by neutrophils or neutrophil-derived supernates are partially antagonized by ketanserin or methysergide, thereby implicating 5-HT as one component of the response. However, there are a number of reasons for concluding that the contractions cannot be attributed solely to 5-HT. In particular, a comparison of the antagonism of 5-HT and neutrophils or neutrophil-derived supernates by ketanserin or methysergide reveals a greater antagonism of the amine than the neutrophil-mediated responses. Endothelial denudation does not modify neutrophil-mediated contractions, whereas it augments those to 5-HT (Cocks & Angus, 1983) and to platelets (Houston *et al.*, 1986). Platelets are the primary source of 5-HT, and, as stated, substantially more platelets are required to elicit similar contractions to those evoked by neutrophils. However, platelet-derived 5-HT may augment the contractions elicited by the neutrophil-derived mediator, as reported for a variety of other vasoconstrictor agents (VanNeuten *et al.*, 1982; Mullane *et al.*, 1982), and could account for a diminution but not abolition of the response to neutrophils.

Another interesting observation is that the neutrophil-derived supernate enhances platelet-induced contractions. Indeed, in the presence of the material from neutrophils, the response to platelets becomes similar to that elicited by the neutrophil suspensions contaminated with platelets (Figure 2). This interaction appears to be synergistic, because stimulating

platelets with A23187 after their addition to the organ baths does not increase further the platelet-induced contraction. A variety of interactions between neutrophils and platelets have been described, where neutrophils or neutrophil-derived mediators promote platelet sequestration and activation (see Mullane, 1989, for review). Consequently the presence of both neutrophils and platelets may be required for full expression of the vasoconstrictor activity.

Extraction of the active principle from neutrophil supernates by cold acetone, but not organic solvents, indicates the mediator is not a lipid, and, therefore, unlikely to be PAF. Because the active principle also co-purifies with other proteins released into the supernates, that is the specific activity of the material increases as the proteins are purified (Figure 7), it may imply that the material is either a protein/peptide, bound to a protein, or co-localized with proteins, perhaps within the granules of the neutrophil.

A review of the literature reveals preliminary reports suggesting neutrophils may influence vascular smooth muscle function (Gillespie *et al.*, 1986; Nishida *et al.*, 1986; Carpenter *et al.*, 1987; Mullane & Sessa, 1988; Rimele *et al.*, 1988). In isolated hearts of rabbits perfused with an artificial buffer containing neutrophils, activation of the cells with PMA produced an increase in coronary perfusion pressure, indicative of coronary vasoconstriction which was ameliorated by free radical scavengers (Gillespie *et al.*, 1986). However, in the isolated heart preparation, coronary perfusion pressure is critically dependent upon left ventricular diastolic pressure, which was also increased by the activated neutrophils. Consequently, it is not clear if the neutrophils enhanced coronary vascular resistance by vasoconstriction or secondary to impaired contractility, which may be due to vascular plugging, perivascular oedema or a direct effect on contractility. In isolated lungs of rats perfused with a neutrophil-containing buffer, the addition of PMA increased pulmonary arterial pressure and vascular permeability (Carpenter *et al.*, 1987). In this preparation the combination of superoxide dismutase and catalase attenuated the vascular permeability changes without reducing the pulmonary arterial pressure, indicating that the increase in pulmonary vascular resistance was not secondary to oedema, and may imply that activated neutrophils evoked pulmonary vasoconstriction. Isolation and identification of specific neutrophil-derived mediators will provide direct evidence to help establish a role for neutrophils in modulating vascular smooth muscle tone.

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