Degranulation enhances release of a stable contractile factor from rabbit polymorphonuclear leukocytes

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Degranulation enhances release of a stable contractile factor from rabbit polymorphonuclear leukocytes. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H1545–H1551, 1998.—We investigated the release of a stable contractile factor(s) from rabbit isolated polymorphonuclear leukocytes (PMNs; 10^8 cells/ml) incubated in Tyrode buffer at 37°C. PMNs were untreated, stimulated with N-formylmethionyl-leucyl-phenylalanine (FMLP; 0.1 µM), or degranulated with cytochalasin B (1 µM) in combination with FMLP (0.1 µM). Products from unstimulated PMNs incubated for 60 min caused significantly greater contraction of rabbit isolated aorta (0.56 ± 0.12 g, n = 8) than did products released from PMNs during a 5-min incubation (0.32 ± 0.07 g, n = 11, P < 0.05). Stimulation alone did not affect contractile factor release; however, products released from degranulated PMNs caused significantly greater aortic contraction (0.48 ± 0.08 g, n = 5) than products from nondegranulated PMNs (0.24 ± 0.04 g, n = 5, P < 0.05) after a 5-min incubation. The contractile activity of PMN-derived products was virtually abolished by heat (90°C, 10 min) or protease (trypsins; 166 U/ml, 5 h) treatment. These findings suggest a PMN-derived protein vasoconstrictor(s) is spontaneously released at a slow rate in vitro and that degranulation can enhance this rate of release. Because PMN degranulation in vivo is associated with inflammation, these results support suggestions that PMN-derived contractile factors may contribute to the impaired blood flow observed during posts ischemic reperfusion.

rabbit thoracic aorta; N-formylmethionyl-leucyl-phenylalanine; cytochalasin B

Ischemia-reperfusion injury initiates an inflammatory response involving infiltration of polymorphonuclear leukocytes (PMNs), and numerous studies indicate that accumulated PMNs contribute to the resultant vascular dysfunction (9, 23, 28, 32). PMN adhesion to vascular endothelium is augmented after ischemia and reperfusion (33), and there is evidence that PMNs contribute to the posts ischemic reduction in blood flow by physically plugging capillaries (9). Recruited PMNs have also been reported to release a number of vasoconstrictor substances that may exacerbate the impaired posts ischemic perfusion, including superoxide anion (25), hydroxyl radical (11), and arachidonic acid metabolites, such as leukotrienes (12) and thromboxane A2 (10). In addition, there have been several reports of the release of stable, proteinlike contractile mediators from PMNs (24, 31, 35).

This study examines the release of a stable substance(s) from peripherally isolated rabbit PMNs that causes endothelium-dependent constriction of rabbit isolated aorta (13, 35) and is also capable of constricting vessels of the rabbit coronary microcirculation (40). Previous studies indicate the factor(s) requires active vessel tone to produce contractions and that those contractions are inhibited by pretreatment of the aorta with the nitric oxide synthase inhibitor N^6-nitro-L-arginine or the nitric oxide scavenger hemoglobin (35). This indicates that the factor(s) mediates contraction by either inhibiting the synthesis of or inactivating the endothelium-derived vasodilator, nitric oxide.

The aim of the present study was to investigate conditions that promote release of this contractile factor(s) because it may be released from PMNs during their accumulation in the vasculature (29). The release of the contractile factor(s) from rabbit PMNs was assayed by the ability of PMN-derived products to contract rabbit isolated aorta. Products released from cells treated with the PMN stimulant N-formylmethionyl-leucyl-phenylalanine (FMLP) were examined to determine the effect of stimulation of the respiratory burst on factor release, whereas FMLP treatment in combination with the degranulation-promoting agent cytochalasin B was used to determine if degranulation of PMN internal storage sites affected release of the contractile factor(s). This study also provides further evidence to suggest the contractile agent(s) under investigation is a protein.

Materials and methods

Tissue and blood collection. New Zealand White rabbits of either sex (2.7 ± 0.2 kg) were anesthetized by intravenous injection of Saffan [4–8 ml, 0.45% alfaxalone and 0.15% alfadalone (wt/vol); Pitman-Moore, North Ryde, Australia]. Anesthetized rabbits were exsanguinated via a polyethylene cannula (0.8 mm ID, 1.2 mm OD) inserted into a carotid artery, with blood drawn into a syringe containing sterile trisodium citrate [0.38% (wt/vol), final concentration]. The rabbit was then killed by an overdose of anesthetic, and the thorax was quickly opened. The thoracic aorta was carefully removed and placed in ice-cold Krebs-bicarbonate solution [composition (in mM) 118.0 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 11.0 d-glucose, 25.0 NaHCO3, and 2.5 CaCl2–2H2O].

Isolation of PMNs. PMNs were isolated under sterile, endotoxin-free conditions, using standard procedures (13, 35, 37). Isolated PMNs were suspended at 10^8 cells/ml in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered Tyrode solution [composition (in mM) 10 HEPES, 137 NaCl, 11.9 NaHCO3, 0.4 KCl, 0.26 MgCl2, and 11 d-glucose, as well as 0.25% (wt/vol) bovine serum albumin (BSA) to maximize contractile activity. In all preparations PMN viability, assessed by trypan blue exclusion, exceeded 90%. A differential cell count was performed on selected preparations stained with a modified Wright-Giemsa stain. The average composition of the leukocyte population was as follows: neutrophils, 91.1 ± 1.0% lymphocytes, 6.9 ± 1.1% eosinophils, 0.9 ± 0.4% monocytes, 0.8 ± 0.2% and basophils, 0.3 ± 0.2% (n = 18).

Incubation of PMN suspensions. Freshly isolated PMNs (10^6 cells/ml) from each animal were divided into two equal...
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volumes, so that cell suspensions were paired in each group, and incubated at 37°C. To investigate the effect of PMN incubation time, we incubated paired cell suspensions for 5 or 60 min. To study the effect of PMN stimulation on factor release, we treated PMNs with FMLP (0.1 µM) or its vehicle (dimethyl sulfoxide, DMSO) and incubated the treated PMNs for either 5 or 60 min. To investigate the effect of PMN stimulation and degranulation on factor release, we treated PMNs with FMLP (0.1 µM) in combination with cytochalasin B (1 µM) or their vehicle (DMSO) and incubated the treated PMNs for either 5 or 60 min (Fig. 1). After all incubations, cell suspensions were centrifuged (5 min, 1,000 g) so that PMN-derived products could be collected as a cell-free supernatant. This supernatant was then stored at −20°C for up to 2 wk before bioassay.

Superoxide anion assay. The release of superoxide anions (O2•−) from PMNs was determined using the superoxide dismutase (SOD)-inhibitable reduction of cytochrome c (17). Before and after each incubation, a PMN sample (2 × 106 cells/ml) was incubated with cytochrome c (80 µM), alone or in combination with either FMLP (0.1 µM) or SOD (30 U/ml) at 37°C for 5 min (Fig. 1). Each reaction was performed in duplicate. Reactions were stopped by centrifugation (5 min, 1,000 g), and the supernatants were decanted and their absorbances were read at 550 nm (Ultronic 2000, Pharmacia Biotech) using the SOD-containing reaction as a blank. O2•− release was calculated using the extinction coefficient (at 550 nm) of 2.1 × 104 M−1 cm−1 and expressed as nanomoles per 106 PMNs.

Myeloperoxidase assay. PMN degranulation was determined with an assay for myeloperoxidase (MPO) (38). PMN-derived supernatant (30 µl) was added to a reaction mixture consisting of tetramethylbenzidine (1.6 mM), sodium phosphate buffer (80 mM, pH 5.4), and H2O2 (0.3 mM) in phosphate-buffered saline (40% vol/vol), preincubated for 2 min at 37°C, and this combination was incubated for a further 3 min at 37°C. The reaction was stopped by addition of sodium acetate buffer (200 mM, pH 3.0) and by placing the mixture on ice. Absorbance of the mixtures was immediately read at 655 nm, and units of MPO activity were expressed as change in absorbance per 106 PMNs per minute. The ability of this treatment to degranulate PMNs has been previously established (1, 38), and in this study, preliminary experiments confirmed that PMNs treated with a combination of FMLP (0.1 µM) and cytochalasin B (1 µM) released a greater amount of MPO [0.18 ± 0.11 U (106 PMN)−1·min−1] than control PMNs [0.02 ± 0.01 U (106 PMN)−1·min−1] in four experiments.

Isolated aortic ring experiments. Rabbit-isolated thoracic aortas were cleared of connective tissue and cut into ring segments ~4 mm in length, which were stored at 4°C in Krebs-bicarbonate solution and used within 24 h of dissection. These segments were mounted in siliconized, 10-ml organ baths containing Krebs-bicarbonate solution maintained at 37°C and continuously bubbled with 95% O2-5% CO2. Aortic ring segments were mounted on two stainless steel hooks, one fixed to a micrometer-adjustable support to allow control of passive tension and the other connected to an isometric force transducer (model FT03, Grass Medical Instruments), and changes in isometric force were recorded on flat-bed chart recorders (model R-02, Rikadenki Kogyo, Tokyo, Japan). Tissues were equilibrated for at least 30 min at 2-g passive tension. Endothelial integrity was then assessed by submaximally contracting the tissues with L-phenylephrine hydrochloride (PE, 1–3 × 10−7 M) before recording the relaxation elicited by acetylcholine perchlorate (ACh, 3 × 10−6 M). Relaxations >70% were taken to indicate a functional endothelium. After drug washout and reequilibration for a minimum of 30 min, rings were again submaximally contracted with PE (10−8 to 10−7 M) to a level matched between groups. Once a stable level of contraction was achieved, PMN-derived supernatant or its vehicle (3–300 µl) was cumulatively added to the organ bath.

Physical characterization of the contractile factor. To examine the possibility that the PMN-derived contractile factor(s) was a protein, we divided PMN supernatants into three equal volumes. One remained untreated, one was heated to 90°C for 10 min, and one was treated with trypsin attached to glass beads (166 U/ml) for 5 h at 37°C. The trypsin-treated PMN supernatants were centrifuged after incubation to remove the bead-bound protease. The supernatant samples were then bioassayed as described above.

Drugs and reagents used. Drugs were obtained and prepared as follows. Trisodium citrate and HEPES were from BDH Chemicals (Poole, UK). Tyrode salts, albumin bovine fraction V (BSA), trypsin (attached to glass beads) were from Sigma (St. Louis, MO). FMLP and cytochalasin B were dissolved and diluted in DMSO. ACh, PE, cytochalasin B, and trypsin (attached to glass beads) were from Sigma (St. Louis, MO). FMLP and cytochalasin B were dissolved and diluted in DMSO. ACh and PE were dissolved in water and further diluted with Krebs solution. Cytochrome c and SOD were dissolved in Tyrode solution and water, respectively.

Statistical Analysis. Results are expressed as means ± SE. PMN-derived supernatant and vehicle curves were analyzed by two-way analysis of variance (ANOVA). Superoxide anion assay data were analyzed by two-tailed paired Student's t-test. Statistical significance was accepted when P < 0.05.

RESULTS

Effect of PMN incubation time. Untreated PMNs (108 cells/ml) were incubated at 37°C for 5 or 60 min. Before
and after each incubation an assay for the FMLP-induced release of $O_2$ was performed on a sample of PMNs to determine if PMNs had been inadvertently stimulated during isolation or incubation, respectively. In all instances FMLP significantly increased $O_2$ release from PMNs over the basal level of release (Fig. 2, inset), indicating that PMNs had not been significantly stimulated before incubation or during the 60-min incubation. Supernatant (3–300 µl) from PMNs incubated for 5 min caused small, but significant, volume-dependent contractions that were significantly enhanced when PMNs were incubated for 60 min (Fig. 2). Thus increasing incubation time increased release of the vasoconstrictor factor(s) without causing stimulation of the PMNs.

Effect of PMN stimulation. The effect of PMN stimulation on vasoconstrictor release was determined with a series of paired experiments where isolated PMNs from each rabbit were separated into two incubates. One was treated with the PMN stimulant FMLP (0.1 µM) and the other with vehicle (DMSO). In one group of experiments these paired cell suspensions were incubated at 37°C for 5 min, whereas in a separate group the suspensions were incubated for 60 min. An assay for FMLP-induced $O_2$ release confirmed that vehicle-treated PMNs remained unstimulated, whereas FMLP-treated PMNs had been effectively stimulated when incubated for either 5 min (Fig. 3A, inset) or 60 min (Fig. 3B, inset). Supernatant (3–300 µl) from vehicle-treated and FMLP-stimulated PMNs produced volume-dependent aortic contractions that were not different when PMNs were incubated for either 5 min (Fig. 3A)
or 60 min (Fig. 3B). Therefore, FMLP-induced PMN stimulation alone did not enhance vasoconstrictor release from PMNs.

Effect of PMN stimulation and degranulation. The effect of PMN degranulation on vasoconstrictor release was investigated in three sets of experiments. Paired PMN suspensions were either vehicle-treated or were treated with FMLP (0.1 µM) and cytochalasin B (1 µM) before incubation at 37°C for 5 min in one group of experiments and 60 min in another. In a separate set of experiments, paired PMN suspensions were both treated with FMLP (0.1 µM) and cytochalasin B (1 µM), with one incubated for 5 min and the other incubated for 60 min, at 37°C. An assay for FMLP-induced $O_2$ release confirmed that, in all cases, vehicle-treated PMNs remained unstimulated, whereas those treated with a combination of FMLP and cytochalasin B had...
PMNs incubated for 5 min (Fig. 4A, inset) or 60 min (Fig. 4B, inset). Supernatant from FMLP-cytochalasin B-treated PMNs caused volume-dependent contractions that were significantly greater than vehicle-treated supernatant when PMNs were incubated for 5 min (Fig. 4A). By contrast, supernatant (3–300 µl) from FMLP-cytochalasin B-treated PMNs produced contractions that were not different when PMNs were incubated for 60 min (Fig. 4B). Interestingly, in this series of experiments, supernatant from PMNs incubated for 60 min (Fig. 4B) produced greater contractions than those induced by supernatant from PMNs incubated for 5 min (Fig. 4A). However, this is a comparison between unpaired samples. Because notable interanimal variation was observed throughout this study, a subsequent experiment was performed in which paired PMN suspensions were both treated with FMLP-cytochalasin B, with one incubated for 5 min and the other for 60 min. In this paired experiment, supernatant from FMLP-cytochalasin B-treated PMNs evoked contractions that were not different when PMNs were incubated for 5 min (maximum contraction 0.71 ± 0.10 g, n = 6) or 60 min (maximum contraction 0.93 ± 0.23, n = 6). Therefore, degranulation enhances vasoconstrictor release during 5 min but not 60 min of incubation. In all experiments, there was no effect of vehicle.

Physical characterization of the contractile factor. Treatment of PMN-derived supernatants with the proteolytic enzyme trypsin (166 U/ml, 5 h) or heating the supernatant to 90°C for 10 min virtually abolished the contractile activity (Fig. 5), suggesting the contractile mediator(s) is a protein.

DISCUSSION

This study confirms that cell-free supernatant from rabbit PMNs causes volume-dependent contraction of precontracted rabbit aorta, as previously reported using both rabbit (13, 35) and human (34) isolated PMNs. These previous studies proposed the observed endothelium-dependent contractions were mediated by a stable, proteinlike factor through a mechanism most likely involving the inactivation of endothelium-derived nitric oxide. The present study examined the release characteristics of this contractile factor(s) from peripherally isolated rabbit PMNs and provides evidence that the factor(s) is a protein. Results from this investigation indicate the factor(s) is spontaneously released from isolated PMNs and that the level of release increases with an increase in the duration of incubation. In addition, it was found that whereas stimulation of PMNs with FMLP alone does not affect factor release, degranulation induced by FMLP in combination with cytochalasin B enhances the rate of release of the PMN-derived vasoconstricor.
Supernatant from untreated PMNs incubated for 60 min caused contractions that were significantly greater than those induced by supernatant derived from PMNs that had been incubated for only 5 min. Importantly, PMNs were not stimulated during isolation or incubation, because FMLP significantly increased the production of O₂⁻ in PMNs before and after each incubation. This indicates a slow release of the contractile substance(s) from unstimulated PMNs. Although it has been suggested that the vasoconstrictor(s) is released without PMN stimulation (13, 35), this report provides the first evidence that the contractile factor(s) is spontaneously released.

Stimulation of PMNs with FMLP alone had no effect on the release of the contractile substance(s), as there was no difference in the level of contraction induced by products released from control PMNs and those stimulated with FMLP when PMNs were incubated for either 5 or 60 min. By contrast, FMLP in combination with the degranulation-promoting agent cytochalasin B enhanced the release of the contractile factor(s) from PMNs over a 5-min incubation. These observations give an insight into possible storage sites of this factor within the leukocyte. PMNs contain four classes of internal storage sites: azurophilic, specific, and gelatinase granules (8, 21, 36), and secretory vesicles (5, 30). These four classes are defined by both their granule content and membrane composition (4, 30); however, secretory vesicles have some distinguishing features. First, secretory vesicles have been reported to contain a number of plasma proteins (2, 3) and are therefore considered endocytic. Second, exocytosis of secretory vesicles is selectively induced by FMLP (4, 22, 29, 30). Because FMLP alone did not affect the release of the PMN-derived contractile factor(s), it is unlikely that the factor(s) mediating the contraction is stored within secretory vesicles. This suggests the vasoconstrictor(s) is not a plasma protein endocytosed by circulating PMNs and released on subsequent stimulation but is synthesized within the cell in which it is stored.

The degranulation-promoting agent cytochalasin B acts by disrupting the cytoskeleton of PMNs, and subsequent stimulation with FMLP has been shown to induce degranulation of all granule classes in both rabbit (39) and human (27) PMNs. This was verified in these studies by an assay for the release of MPO, a specific marker of PMN degranulation (14, 39). The finding that degranulation enhances contractions to PMN-derived supernatant when PMNs are incubated for 5 min suggests that the contractile factor(s) is stored within specific, azurophilic, or gelatinase granules. The subcellular localization of the contractile factor(s) is of interest because it has been proposed that during an inflammatory response, specific granules of accumulated PMNs degranulate within the vasculature, whereas the contents of azurophilic granules are not released until PMNs have migrated to within the tissue (4). If this is the case, the contractile substance(s) under investigation in this study is more likely to contribute to the postischemic reduction in blood flow observed in ischemia-reperfusion injury if it is stored within azurophilic granules, where the dilutional effects of blood flow will not be a factor. It is important to note, however, that localization of the factor(s) may not be restricted to a single granule type as several PMN proteins have been localized in more than one granule type. For example, the degradative enzyme gelatinase is often considered a marker for gelatinase granules; however, it has also been identified in specific granules (19). Lysozyme has been localized in both specific and azurophilic granules (6).

Interestingly, whereas degranulation enhanced contractile responses to supernatant from PMNs incubated for 5 min, no such enhancement was observed when cells were incubated for 60 min. The current hypothesis for this observation relies on the assumption that, over a 60-min period, maximal or near-maximal amounts of stored contractile activity are released from unstimulated PMNs. If this is the case, then it may be that the enhanced release induced by degranulation over a 5-min incubation is masked after a 60-min incubation. This hypothesis is supported by the further observation in this study that there is no difference between the level of contraction caused by supernatants from PMNs that have undergone degranulation for either 5 or 60 min. This contrasts with contractions induced by supernatant from unstimulated cells, in which supernatant from a 60-min incubation resulted in a greater level of contraction than that from a 5-min incubation. Therefore, degranulation appears to increase the rate of contractile factor(s) release so that maximal release by degranulation occurs within ~5 min, whereas spontaneous release from unstimulated PMNs occurs more slowly, with maximal release occurring within 60 min.

It is interesting that vasoconstrictor release from intracellular granules occurs without stimulation; however, this spontaneous release mechanism may be analogous to the spontaneous transmitter release from neuronal cells (18). It has been postulated that the constant fluctuations in intracellular calcium concentration are responsible for this unstimulated release, given that a rise in cytosolic calcium concentration is essential for exocytosis from neurons (7). Because there is substantial evidence suggesting that calcium has a similar role in the exocytosis of PMN granules (20, 26, 30), a spontaneous release mechanism analogous to that observed in neuronal cells may be occurring in PMNs. Alternatively, it is possible that the spontaneous release of contractile factor from PMNs observed during the in vitro conditions in this investigation does not occur in vivo because the isolation procedures affect cellular integrity. If this were the case, we would expect circulating PMNs not to release stored vasoconstrictor but for release to be induced on PMN accumulation and subsequent degranulation at an inflammatory site. Unfortunately, investigating the release of PMN-derived products in vivo is a difficult task, and it remains to be seen under what conditions this factor is released in vivo. However, recent reports by Jin and colleagues (15, 16) provide evidence for the endogenous release of a vasoconstrictor substance that appears to
act through a mechanism similar to the factor(s) under investigation in the present study. They report that a stable factor capable of inhibiting nitric oxide synthase is released into the plasma of ischemic rats and may cause vasoconstriction. It is possible that this factor is released into plasma by PMNs that have accumulated at sites of ischemic vascular injury and have degraded. The findings of in et al. (15, 16) in combination with the findings of this report suggest that further investigation into the origin and physiological relevance of the contractile factors involved is warranted because they may play important roles in the pathogenesis of the sustained reduction in blood flow associated with reperfusion of previously ischemic vascular beds.

It has been suggested that the contractile factor(s) under investigation is a protein because it is stable and has an estimated size of >5 kDa (34). The findings of this study, that the contractile activity of PMN-derived products is sensitive to protease or heat treatment, supports this proposal. In addition, the size of the contractile factor(s) has been estimated at 30 kDa using gel chromatography (Hart and Woodman, unpublished observations). Clearly, these findings distinguish this substance(s) from small, labile PMN-derived contractile mediators, such as arachidonic acid metabolites and oxygen radicals. The protein contractile factor(s) is also unlikely to stimulate the production of such mediators, as indomethacin has no effect on contractile responses to PMN supernatant (40).

In summary, the results of this investigation support previous reports that rabbit isolated PMNs release a stable vasoconstrictor substance(s) capable of causing concentration-dependent contraction of precontracted rabbit aorta. Furthermore, this study also provides evidence that this substance(s) is a protein stored within azurophilic, specific, or gelatinase granules of PMNs. Under the in vitro conditions of this study the factor(s) can be either slowly released from unstimulated PMNs or released more rapidly on degranulation. Consequently, this protein vasoconstrictor(s) appears to be synthesized and stored within rabbit PMNs and may be released under the conditions associated with PMN accumulation and degranulation in inflammatory conditions such as myocardial ischemia and reperfusion.

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REFERENCES


22. Lundquist, H., M. Gustafsson, A. Johansson, E. Sardnald, and C. Dahlgren. Neutrophil control of formylmethionyl-leucyl-phenylalanine induced mobilisation of secretory vesicles and...


