Pharmacological Potentiation of Natriuretic Peptide Limits Polymorphonuclear Neutrophil–Vascular Cell Interactions

El Mostafa Mtairag, Xavier Houard, Samira Rais, Catherine Pasquier, Mounia Oudghiri, Marie-Paule Jacob, Olivier Meilhac, Jean-Baptiste Michel

Objective—Activated polymorphonuclear neutrophils (PMNs) are the main source of circulating neutral endopeptidase (NEP). We tested the hypothesis that NEP inhibition could potentiate the effect of atrial natriuretic peptide (ANP) on PMN–vascular cell interactions in vitro.

Methods and Results—ANP alone and its potentiation by retrothiorphan, the NEP inhibitor, significantly inhibited superoxide, lysozyme, and matrix metalloproteinase (MMP)-9 release by N-formyl-Met-Leu-Phe–stimulated PMNs. Activated PMNs degraded exogenous ANP, which was prevented by NEP inhibition. Hypoxia significantly increased the adhesion of PMNs to endothelial cells and their subsequent MMP-9 release by 60% and 150%, respectively (P<0.01). ANP and its potentiation by retrothiorphan limited PMN adhesion to hypoxic endothelial cells and thus decreased their MMP-9 release (P<0.01). Smooth muscle cells (SMCs) incubated with conditioned medium of N-formyl-Met-Leu-Phe–stimulated PMNs exhibited morphological and biochemical changes characteristic of apoptosis (terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling positivity, nuclear condensation/fragmentation, poly ADP-ribose polymerase cleavage, and DNA laddering). SMC detachment and subsequent apoptosis could be related to leukocyte elastase–induced pericellular proteolysis, inasmuch as both events are inhibited by elastase inhibitors. ANP and its potentiation by retrothiorphan were able to limit elastase release, fibronectin degradation, and SMC apoptosis.

Conclusions—ANP potentiation by NEP inhibition could limit PMN activation and its consequences on vascular cells.


Key Words: neutral endopeptidase inhibitors ■ endothelial cells ■ smooth muscle cells ■ proteases ■ anoikis

The interactions between polymorphonuclear neutrophils (PMNs) and vascular cells play an important role in vascular pathology. By their ability to adhere to hypoxic endothelium1 and to release proteinases,2 PMNs could injure vascular cells by pericellular proteolysis.3 Natriuretic peptides are mainly metabolized by clearance receptors and by neutral endopeptidase (NEP),4 a zinc ectopeptidase. Few studies have addressed the role of NEP in the degradation of atrial natriuretic peptide (ANP) in the circulation. PMNs represent the main source of NEP5,6 in the circulation and, therefore, largely contribute to natriuretic peptide degradation in the plasma.5 PMNs express particulate guanylate cyclase A, the active receptor for ANP and brain natriuretic peptide,7 which limits PMN activation by the generation of intracellular cGMP.8 Nevertheless, the effects of natriuretic peptides on PMNs remain controversial. Wiedermann et al9 reported a potentiating effect of ANP on N-formyl-Met-Leu-Phe (fMLP)-induced superoxide release. Similar results with brain natriuretic peptide were reported by Garlichs et al10 and by Biselli et al11 with the use of leukotriene B4 as a trigger. In contrast, Matsumura et al10 showed that ANP and its potentiation by NEP inhibition increased intracellular cGMP, which limited protease release by PMNs.

Therefore, we hypothesized that the potentiation of ANP by NEP inhibition could limit PMN–vascular cell interactions by acting predominantly on PMNs. For this purpose, we have developed an in vitro approach testing the ability of ANP potentiation by NEP inhibition (1) to inhibit PMN protease release, (2) to limit PMN–endothelial cell interactions in hypoxic conditions, and (3) to limit the pericellular proteolysis induced by activated PMN secretions.

Methods

Cell Cultures

Human umbilical vein endothelial cells (HUVECs) were harvested and cultured as described.12 Human smooth muscle cells (SMCs) were obtained from venous wall explants after removal of the adventitia and were cultured.13
Isolation of Human PMNs
PMNs were obtained from heparinized venous blood by Ficoll-Paque (Amersham) centrifugation, followed by 2% dextran sedimentation and hypo-osmotic lysis of erythrocytes, and they were maintained in HBSS buffer. The viability, measured by the release of lactate dehydrogenase, and purity of the final preparation were >99% and 95%, respectively.

Degranulation and Oxidative Burst Studies
fMLP at 10⁻⁶ mol/L was used as a stimulant of PMN degranulation and superoxide anion production. Lysozyme release was measured by Micrococcus lysodeikticus lysis, spectrophotometrically monitored at 450 nm, and expressed as the percentage of total cellular activity. Matrix metalloproteinase (MMP)-9 was detected by gelatin zymography. Superoxide anion generation was measured by the superoxide dismutase–inhibitable reduction of cytochrome c.

ANP Radioimmunoassay
Supernatants from PMNs preconditioned with ANP (10⁻⁸, 10⁻⁹, and 10⁻¹⁰ mol/L) combined or not with retrothiorphan, the NEP inhibitor (NEPI), at 10⁻⁶ mol/L were subjected to radioimmunoassay as described.

Endothelial Cell Hypoxia
Deprived HUVECs (second or third passage) were exposed for 150 minutes to a hypoxic environment (2% O₂/98% N₂). Medium was removed, and hypoxic HUVECs were immediately subjected to binding assays.

PMN Labeling and Binding Assays
Binding assays were performed by using an entrapped self-quenching fluorescent dye, calcine-AM. PMNs (10×10⁶ cells/mL) were incubated with 2.5 μmol/L calcine-AM for 30 minutes at 37°C. Cells were then washed, and labeled PMNs were resuspended.

Fluorescent PMNs (10⁶/mL HBSS) were preconditioned with increasing ANP concentrations (10⁻⁸, 10⁻⁹, and 10⁻¹⁰ mol/L) for 10 minutes at 37°C without or with NEPI (10⁻⁶ mol/L) and were then added to either normoxic or hypoxic HUVECs for 15 minutes at 37°C. The culture medium was collected, centrifuged at 400g, and subjected to gelatin zymography. The adherent cells were washed, and fluorescence was quenched on a Fluostar plate reader (LabTechnologies) with the use of 480- and 520-nm wavelengths for excitation and emission, respectively. Results were expressed as a percentage of the fluorescence measured in PMN/normoxic HUVEC coculture. The assay was highly reproducible, and the PMN number was directly proportional to fluorescence with a positive correlation (r=0.97).

SMC Treatment
PMNs (10⁶ cells) were suspended in serum-free RPMI and preconditioned with ANP (10⁻⁷ mol/L) alone or combined with NEPI (10⁻⁶ mol/L) for 10 minutes at 37°C. They were then stimulated with fMLP (10⁻⁶ mol/L) for 5 minutes. This PMN-conditioned supernatant was collected and centrifuged at 400g for 5 minutes before addition to SMCs. Confluent SMCs (second or third passage) were serum-deprived and incubated at 37°C with 1 mL PMN-conditioned supernatant for 24 hours. Culture medium was collected and centrifuged at 400g for 5 minutes and was then subjected to casein and gelatin zymography or Western blot analysis. Protein concentrations were determined by using the Bradford assay (Bio-Rad).

Evaluation of Apoptosis
Cell Detachment and Death
Adherent SMCs were assessed by the MTT test. Cell death detection was performed by photometric enzyme immunoassay for in vitro determination of cytoplasmic histone–associated DNA fragments (Roche). Caspase-3 activity was assessed by a kit using a specific substrate (DVED-pNA, R&D Systems). Detached SMCs were cytocentrifuged at 150g for 6 minutes onto slides and fixed.

Adherent and detached cells were stained with hematoxylin and eosin or subjected to terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL), which was used to visualize DNA fragmentation in situ. A positive control (1 μg/mL DNase) and a negative control (without terminal transferase) were included. DAPI staining allowed us to visualize all nuclei. Genomic DNA was isolated from SMCs by the use of standard DNA extraction methods (G-NOME kit, B10101). DNA (10 μg) was loaded on a 1.8% agarose gel containing 0.5 μg/mL ethidium bromide and separated by electrophoresis.

SDS-PAGE Zymography
Gelatinolytic and caseinolytic activities in cell culture media were measured as previously described. Samples containing 5 and 15 μg of proteins were loaded on appropriate gelatin and casein gels. Lysis areas were quantified by densitometric scanning. To discriminate between MMP and serine protease activity, additional gels prepared in the presence of EDTA (30 mmol/L) or Pefabloc (2 mmol/L, VWR) were loaded. To investigate the nature of the caseinolytic activity, Eglin c and Elafin (Sigma Chemical Co) and α₁-antitrypsin (Calbiochem), known to inhibit elastase activity, were added to the cocultures.

Western Blot
Standard procedures were used. Poly ADP-ribose polymerase (PARP) and fibronectin were detected in cells and culture media, respectively (n=3 experiments), with the use of SDS-PAGE, and proteins were transferred to membranes (Millipore). The membranes were blocked with anti-PARP or anti-fibronectin (Ab-1, Calbiochem; 1:2000 dilution) antibodies and a peroxidase-conjugated secondary antibody. Membranes were then exposed to x-ray films after chemiluminescence detection.

Figure 1. PMN function. Shown is the effect of ANP alone or ANP + NEPI on lysozyme release and superoxide anion production by PMNs stimulated with fMLP (10⁻⁶ mol/L) for 5 minutes. Bars represent activities detected in the supernatant, which are expressed as a percentage of total cellular activity (100%). There were 5 independent experiments. *P<0.01 vs control; #P<0.01 vs ANP effect.
Statistical Analysis
Data are presented as mean±SD. Comparisons were performed by a 1-way ANOVA, followed by the Fisher test when appropriate. A value of $P<0.01$ was considered statistically significant.

Results

ANP Directly Prevented PMN Activation

PMN Degranulation
Lysozyme release by PMNs in response to fMLP was 46±5.3% of total cellular activity ($n=5$, $P<0.01$). When PMNs were preconditioned with ANP and then stimulated with fMLP, lysozyme release significantly decreased in a dose-dependent manner (Figure 1A). This attenuation was significantly enhanced in the presence of 10-8 mol/L NEPI. ANP alone or combined with NEPI had no effect on lysozyme release by unstimulated PMNs (data not shown). PMN preconditioning with NEPI alone did not modify fMLP-stimulated PMN degranulation, which was 95±2.3% of the value for fMLP-stimulated PMNs.

Respiratory Burst Function
Figure 1B shows respiratory burst function. In response to fMLP, PMNs generated 10±3.1 nmol/106 cells of superoxide anion ($n=5$, $P<0.001$). Preconditioning of PMNs with ANP significantly reduced this response in a dose-dependent manner. NEPI significantly potentiated this inhibition. ANP alone or combined with NEPI had no effect on superoxide anion generation by unstimulated PMNs. PMN preconditioning with NEPI alone did not modify the fMLP-stimulated PMN oxidative burst, which was 96±1.6% of the value for fMLP-stimulated PMNs.

ANP Degradation
fMLP-stimulated PMNs significantly degraded exogenous ANP at all concentrations used ($P<0.001$). NEPI protected ANP from degradation by fMLP-stimulated PMNs. Please see online Figure I, available at http://www.ahajournals.org.

Gelatinase B Release
Figure 2 shows gelatinase B release. Unstimulated PMNs show basal release of pro-MMP-9 at 92 kDa as monomers, 220 kDa as disulfide-linked homodimers, and 135 kDa as heterodimer complexes of 92-kDa gelatinase and the 25-kDa lipocalin. Compared with no stimulation, stimulation of PMNs with fMLP significantly increased MMP-9 release into the medium (first column). There were 5 experiments. $^*P<0.01$ vs unstimulated PMNs; $#P<0.01$ vs fMLP-stimulated PMNs.

PMN–Endothelial Cell Interactions

Effect of Hypoxia on PMN Adhesion to HUVECs
The basal adhesion level of PMNs to normoxic HUVECs (20% O2), considered as 100%, was 40 000±8500 cells for 107 PMNs. PMNs became significantly more adherent to hypoxic HUVECs, with 64 000±4300 adhering cells ($P<0.01$, Figure 3A).

Effect of ANP Potentiation on PMN Adhesion
Figure 3A shows the effect of ANP potentiation on PMN adhesion. Preconditioning by ANP resulted in a significant dose-dependent inhibition of PMN adhesion to hypoxic HUVECs ($P<0.01$), which returned to baseline normoxic levels at 10-7 mol/L. When ANP preconditioning was combined with NEPI, the inhibitory effect was significantly accentuated for all ANP concentrations ($P<0.01$). No significant effect of ANP alone or ANP+NEPI was observed on PMN/normoxic HUVEC interactions (data not shown). PMN preconditioning by NEPI alone did not modify PMN adhesion to hypoxic HUVECs, with 64 000±7000 adhering cells.

PMN Degranulation
Figure 3B shows PMN degranulation. MMP-9 was not detected in HUVECs;12 Small amounts of MMP-9 were spontaneously released from isolated PMNs (Figure 2), and these were not modified by PMN–normoxic HUVEC cocultures in the absence or presence of ANP or NEPI (data not shown). PMN adhesion to hypoxic HUVECs significantly enhanced MMP-9 release into the medium ($P<0.01$). The addition of ANP resulted in a dose-dependent decrease in MMP-9 release ($P<0.01$), which was further inhibited by NEPI ($P<0.01$).

PMN-SMC Interactions

Effect of PMN Supernatant on SMCs
Whereas the supernatant of unstimulated PMNs had no effect on SMC cultures (Figure 4A2), the 24-hour incubation of
SMCs with supernatant from fMLP-stimulated PMNs induced cell retraction and TUNEL positivity (Figure 4A3 and 4A6). The addition of NEPI potentiated the protective effect of ANP; similar protection against apoptosis was obtained with elastase inhibitors (data not shown).

Supernatant of fMLP-stimulated PMNs induced a detachment of SMCs, which presented typical apoptosis features, ie, nuclear and DNA condensation/fragmentation (Figure 4A5, 4A6, and 4B2), whereas adherent SMCs alone or cultured with supernatant from unstimulated PMNs or from PMNs preconditioned with ANP exhibited a normal morphology (Figure 4B1). The morphological hallmarks of apoptosis observed in SMCs were accompanied by DNA laddering, as detected by DNA electrophoresis (Figure 4C), and PARP cleavage (please see online Figure II, available at http://www.ahajournals.org). ANP and NEP inhibition prevented DNA fragmentation and PARP cleavage.

Incubation of SMCs in the supernatant of fMLP-stimulated PMNs markedly induced caspase-3 activation and DNA fragmentation (Figure 5A and 5B). Both apoptotic parameters were prevented by ANP alone or ANP+NEPI and also by elastase inhibitors ($P<0.001$). The MTT test showed that the supernatant from fMLP-stimulated PMNs induced SMC detachment (Figure 5B), which was inhibited by ANP ($10^{-7}$ mol/L) alone and more markedly by ANP+NEPI as well as by elastase inhibitors.

**Elastase Activity**

Figure 6A shows elastase activity. This activity was not detected in SMC culture alone, with or without ANP and NEPI (data not shown). The supernatant of unstimulated
PMNs showed a basal release of elastase detected at 29 kDa by its caseinolytic activity. In contrast, conditioned medium from SMCs incubated with supernatant of fMLP-stimulated PMNs showed an ~13-fold enhancement of elastase activity, which was reduced to 8- and 5-fold by preconditioning with ANP alone or ANP+NEPI, respectively. Elastase inhibitors reduced the caseinolytic activity to baseline levels.

Fibronectin Degradation
Figure 6B shows fibronectin degradation. In SMCs and PMN-conditioned media, limited fibronectin degradation products were detected by Western blot analysis. In contrast, SMCs cultured with the supernatant of fMLP-stimulated PMNs showed important fibronectin degradation, with the production of small fragments ranging from 220 to 40 kDa. The addition of ANP alone or ANP+NEPI prevented fibronectin degradation. Similarly, elastase inhibitors prevented fibronectin degradation.

Discussion
PMN–vascular wall interactions may play a pathological role in different situations, including ischemia/reperfusion injury, blood stasis–induced varicose vein development, and a role for PMNs in plaque rupture and aneurysm development has also been suggested. In these different situations, the interaction involves mainly PMN adhesion to the stimulated endothelium, followed by the activation of PMNs, including an oxidative burst and the release of granule contents. In a first step, we studied the ability of ANP and its potentiation by the NEPI to limit PMN activation. We used fMLP, a classic extracellular compound able to activate PMNs, to induce the oxidative burst and the release of granule contents. Latent MMP-9, which is easy to measure through its gelatinolytic activity by zymography, can be used as an early and sensitive marker of PMN activation in vivo because...
pro-MMP-9 is contained in gelatinase granules and is readily mobilized. In humans, plasma levels of MMP-9 have been used as a marker for abdominal aneurysms and for blood stasis in varicose veins.

fMLP activates PMNs through binding to a 7-transmembrane-domain receptor that induces calcium mobilization and protein kinase C activation, leading to the oxidative burst and granule release. As in other cell types, the ANP-induced generation of cGMP probably acts at different levels of the intracellular signaling process, desensitizing PMNs to activator agents. The expression of particulate guanylate cyclase and ectoexposition of NEP by activated PMNs may account for the observation that ANP limits fMLP-induced PMN activation and that retrothiorphan potentiates this inhibitory effect.

Hypoxia is a pathological condition in which PMNs are activated by contact with endothelial cells. In the present study, we show that hypoxia of endothelial cells not only increased PMN adhesion but also induced a significant increase in MMP-9 release by PMNs, providing evidence of a process of activation and degranulation. ANP was able to limit PMN adhesion to hypoxic endothelial cells and MMP-9 release, and NEP inhibition potentiated this effect. These data suggest that MMP-9 release could be used as an early and sensitive marker of PMN activation in vivo but also as an intermediate soluble marker to test the therapeutic efficiency of drugs in clinical situations involving pathological PMN–endothelial cell interactions. Our data do not fit well with the results recently published by Izumi et al, who showed a beneficial effect of the blockade of particulate guanylate cyclase A in models of ischemia/reperfusion in mice. Nevertheless, the authors focused on reoxygenation and on the potentiating effect of ANP on oxidative stress–induced endothelial cell activation. The direct effect of ANP on PMNs was not investigated. In contrast, the present study focused on the direct effect of ANP on PMNs.

We also tested the interaction between PMN granule release and SMC survival. Some vascular pathologies involve smooth muscle disappearance. In the vascular wall, SMCs secrete and mature their own extracellular matrix, such as fibronectin, an adhesive protein to which they adhere via the integrin system. Leukocyte elastase has been shown to proteolyse fibronectin, generating soluble fragments. In parallel, anchorage to extracellular matrix is necessary for the survival of adherent cells. Indeed, it has been shown that loss of adhesion induces cell apoptosis, a phenomenon termed anoikis.

Our data suggest that SMC anoikis occurs, at least in part, because of degradation of the pericellular adhesive protein, as demonstrated by the solubilization of fibronectin fragments in the culture medium. Recently, it has been demonstrated that serum deprivation leads to apoptosis of cultured SMCs. This phenomenon is associated with cellular fibronectin degradation and is prevented by antiprotease addition. Nevertheless, proteolysis of other pericellular adhesive proteins, such as laminin, also sensitive to leukocyte elastase, may occur. We also demonstrated that serine proteases are probably the main molecular effectors of SMC anoikis. We detected leukocyte elastase through its caseino-
lytic activity and demonstrated that inhibitors of leukocyte elastase\textsuperscript{24–24,42,43} were able to inhibit the casemolytic activity of activated PMN-conditioned medium, to limit fibronectin degradation, and to prevent SMC retraction and anoikis, suggesting that leukocyte elastase is the main molecular effector of the phenomenon. Nevertheless, other agents, such as external superoxide anion $O_2^-$ can induce apoptosis in some cultured cells, eg, arabidopsis mutants\textsuperscript{44} and cardiomyocytes.\textsuperscript{45}

Finally, we tested the hypothesis that ANP potentiation by NEP inhibition was able to prevent SMC anoikis induced by activated PMN supernatant. Preconditioning PMNs with ANP and NEP inhibition limited the ability of this supernatant to generate casemolytic activity, to solubilize fibronectin fragments, and to induce SMC anoikis. Recently, it has been shown that ANP overexpression in SMCs reduces cell growth and induces anoikis.\textsuperscript{46} Of interest, exogenous ANP did not induce SMC apoptosis in that study, in accordance with our results, and the authors suggested that the ANP accumulation within the cell triggered apoptosis.

In conclusion, the present study confirms that natriuretic peptide and NEP inhibition can limit PMN activation. We demonstrated that ANP potentiation by NEP inhibition was able to inhibit PMN adhesion to hypoxic endothelial cells and the subsequent release of latent MMP-9, suggesting that latent MMP-9 could be used as an intermediate marker of PMN activation. The present study demonstrates, for the first time, that leukocyte elastase released by activated PMNs induces SMC anoikis, which is due, at least in part, to pericellular proteolysis of fibronectin. This phenomenon is also limited by ANP and by NEP inhibition. These data suggest that pathological conditions involving activated PMN–vascular cell interactions could be interesting therapeutic targets for NEP inhibition in vivo.

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References


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Legends

**Figure I: ANP degradation.** ANP degradation by PMNs. n=3 experiments; **p<0.001 vs.** unstimulated PMNs pre-incubated with ANP.

**Figure II: Western blot of PARP.** PARP cleavage in SMCs.
C

ANP (log nM)

PMN - - - +++++ +++++ +++++
fMLP (10^{-6} M) - - - -++++ +++++
NEPI (10^{-6} M) - - - - - - - ++++
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