

Inhibition of p38 MAPK Activation via Induction of MKP-1

Atrial Natriuretic Peptide Reduces TNF- α -Induced Actin Polymerization and Endothelial Permeability

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Abstract—The atrial natriuretic peptide (ANP) is a cardiovascular hormone possessing antiinflammatory potential due to its inhibitory action on the production of inflammatory mediators, such as tumor necrosis factor- α (TNF- α). The aim of this study was to determine whether ANP is able to attenuate inflammatory effects of TNF- α on target cells. Human umbilical vein endothelial cells (HUVECs) were treated with TNF- α in the presence or absence of ANP. Changes in permeability, cytoskeletal alterations, phosphorylation of p38 MAPK and HSP27, and expression of MKP-1 were determined by macromolecule permeability assay, fluorescence labeling, RT-PCR, and immunoblotting. Antisense studies were done by transfecting cells with MKP-1 antisense oligonucleotides. Activation of HUVECs with TNF- α lead to a significant increase of macromolecule permeability and formation of stress fibers. Treatment of cells with ANP (10^{-8} to 10^{-6} mol/L) significantly reduced the formation of stress fibers and elevated permeability. Both TNF- α -induced effects were shown to be mediated via the activation of p38 using SB203580, a specific inhibitor of p38. ANP significantly reduced the TNF- α -induced activation of p38 and attenuated the phosphorylation of HSP27, a central target downstream of p38. ANP showed no effect on p38 upstream kinases MKK3/6. However, a significant induction of the MAPK phosphatase MKP-1 mRNA and protein could be observed in ANP-treated cells. Antisense experiments proved a causal role for MKP-1 induction in the ANP-mediated inhibition of p38. These data show the inhibitory action of ANP on TNF- α -induced changes in endothelial cytoskeleton and macromolecule permeability involving an MKP-1-induced inactivation of p38 MAPK. These effects point to an antiinflammatory and antiatherogenic potential of this cardiovascular hormone. (*Circ Res.* 2002;90:874-881.)

Key Words: signal transduction ■ inflammation ■ endothelium ■ hormones ■ natriuretic peptides

Tumor necrosis factor- α (TNF- α) is one of the primary inflammatory cytokines associated with developing atherosclerotic lesions. It is mainly produced by activated monocytes and macrophages and influences the growth and behavior of endothelial cells, monocytes, and smooth muscle cells.¹ TNF- α exerts several effects that facilitate the formation of an atheromatous plaque: it increases the expression of endothelial cell adhesion molecules, induces proliferation of smooth muscle cells, and increases endothelial cell leakiness. Formation of intercellular gaps in vascular endothelium is regarded as one of the initial conditions contributing to the development of an atheromatous plaque.² An increased vascular permeability is commonly attributed to the reorganization of F-actin filaments followed by contraction of cells and formation of intercellular gaps.³⁻⁶

The TNF- α -induced reorganization of F-actin, ie, the formation of stress fibers, is associated with the polymerization of G-actin into F-actin fibers.⁴ Heat shock protein HSP27 has been closely associated with the regulation of actin

polymerization⁷: phosphorylated HSP27 has been shown to significantly stabilize F-actin and thereby to increase fiber formation.^{7,8} HSP27 is phosphorylated by the mitogen-activated kinase-activated protein kinase-2 (MAPKAPK-2) and therefore represents a downstream target of the p38 mitogen-activated protein kinase (MAPK) (for review see Obata et al⁹). p38 MAPK itself is activated by the MAPK kinases 3 and 6 (MKK3/6). Besides the regulation of p38 phosphorylation by its upstream kinases, inactivation of MAPK in mammalian cells is achieved by a family of dual-specificity MAPK phosphatases (MKP), which target the 2 critical phosphorylation sites in the activation loop of MAPK. From the known MKPs, MKP-1 is known to be responsible for the dephosphorylation of p38,¹⁰ which could not be shown for MKP-2¹¹ or MKP-3.¹²

Recently, we and others could demonstrate that a cardiovascular hormone, the atrial natriuretic peptide (ANP), attenuates production of TNF- α in macrophages.^{13,14} The aim of this study was to investigate whether ANP also influences

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effects of TNF- α exerted on respective target cells, such as endothelial cells.

ANP was discovered when the group of de Bold¹⁵ observed that injection of atrial extracts into rats leads to a massive natriuresis and diuresis in these animals. Besides the kidney, the vascular system has been shown to be affected by this peptide hormone. ANP possesses vasodilating properties and thus plays an important role in the regulation of blood pressure.¹⁶ ANP mainly acts through binding to the guanylyl cyclase-coupled A-receptor (NPR-A) and thus production of cGMP.^{16,17}

ANP expression and function, however, are not restricted to the cardiovascular system. ANP and its receptors have been shown to be expressed in cells and tissue of the immune system, such as thymus¹⁸ or macrophages.^{19–21} The peptide was demonstrated to inhibit thymocyte proliferation²² and thymopoiesis.²³ Moreover, ANP seems to exert antiinflammatory action because ANP was shown to attenuate the induction of iNOS, a central proinflammatory enzyme, in an autocrine fashion.^{21,24–26} ANP also attenuates the induction of other inflammatory mediators, such as cyclooxygenase-2²⁷ and TNF- α .^{13,14} The latter was demonstrated in LPS-stimulated macrophages¹³ as well as in reperfused livers.²⁸ Because ANP was shown to exert profound cytoprotective action,^{29–31} it was hypothesized that ANP does not only reduce the production of inflammatory mediators but also suppresses their action. In fact, in the present study, we demonstrate a beneficial effect of ANP on TNF- α -induced inflammatory processes in endothelial cells and elucidate the underlying molecular mechanisms.

Materials and Methods

Materials

ANP and anti-p38 were from Calbiochem; medium and penicillin/streptomycin were from PAN Biotech; FCS was from Biochrom; chemoluminescence kit was from NEN; Complete was from Roche; phosphospecific antibodies were from Cell Signaling; anti-MKP-1 and anti-HSP27 were from Santa Cruz; and peroxidase-conjugated goat anti-rabbit and donkey anti-goat were from Jackson Immunolab (Dianova, Hamburg, Germany). Rhodamine-conjugated phalloidin was from Molecular probes-Mobitec. All other materials were purchased from either Sigma or Merck-Eurolab.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were prepared by digestion of umbilical veins with collagenase and grown in M199 containing 20% FCS, endothelial cell supplement (Sigma), and penicillin/streptomycin. In order to compensate for interindividual differences, cells of at least 2 umbilical cords were combined in each cell preparation. For experiments, cells of passage No. 3 or 4 were grown until confluence. HUVECs were found >95% pure as judged by FACS analysis, using an antiserum against the von Willebrand factor.

Permeability Assay

HUVECs were cultured in transwell plates (0.4 μ m, Costar). Cells were either left untreated or stimulated with TNF- α (Sigma) with or without ANP or SB203580, added to the cells 30 minutes before TNF- α . After 24 hours, fluorescein-isothiocyanate-labeled bovine serum albumin (BSA) (FITC-BSA, 10 mg/mL, Sigma) was given to the cells. Cell culture medium from the lower compartment was removed after 60 minutes. FITC-BSA was quantified in a fluorescence spectrofluorophotometer. BSA flux is expressed as ratio

between fluorescence intensities in the lower compartment (60 minutes) and the upper compartment (0 minutes).

Morphological Investigations

HUVECs were either left untreated or stimulated with TNF- α in the presence or absence of ANP, which was added to the cells 30 minutes before TNF- α . After 1 or 24 hours, cells were stained with HEMAcolor. Cells were photographed with a Zeiss Axioskop MC 80 DX microscope.

Actin Staining

HUVECs were either left untreated or stimulated with TNF- α for 30 minutes in the presence or absence of ANP or SB203580 (Calbiochem). ANP and SB203580 were added to the cells 30 minutes before TNF- α . Washed cells fixed with paraformaldehyde washed again and permeabilized for 5 minutes with 0.1% Triton X-100. Washed cells were incubated with a 1% solution of BSA (30 minutes, RT), and stained with rhodamine-phalloidin (0.16 mol/L, 20 minutes, 4°C, darkness). Stained F-actin was visualized using a Zeiss Axiovert 25 microscope with a 200- or 400-fold magnification.

Quantification of F-Actin

HUVECs were left either untreated or treated with TNF- α . The effect of ANP, SB203580, and 8-Br-cGMP alone or in combination with TNF- α on F-actin formation was determined. MTT assay³² showed that ANP, SB203580, and 8-Br-cGMP in the used concentrations do not have cytotoxic activity on HUVECs (data not shown). Substances were added to the cells 30 minutes before TNF- α . F-actin staining was performed as described above. Bound dye was extracted from the cells with methanol. The fluorescence of the methanolic dye solution was measured in a Shimadzu, RF 1501 spectrofluorophotometer. The mean fluorescence of TNF- α -treated cells was set as 100% and all other treatment conditions were compared with this group.

Western Blot

HUVECs were either left untreated or stimulated with TNF- α in the presence or absence of ANP, which was added to the cells 30 minutes before TNF- α . Western blots were performed according to Kiemer and Vollmar,²⁶ whereby lysis buffer additionally contained (in mmol/L) 5 Na-pyrophosphate, 1 PMSF, 50 NaF, and 50 sodium vanadate. Enhanced chemoluminescence and a Kodak Image station (Kodak Digital Science) were used for visualization of the bands.

In Vitro Kinase Activity

HUVECs were either left untreated or treated with TNF- α in the presence or absence of ANP. After 10 minutes, cells were washed, lysed, and centrifuged. Protein (300 μ g) was immunoprecipitated with an anti-p38 antibody and protein A agarose. Immunoprecipitates were resuspended in kinase buffer containing myelin basic protein (MBP) as a substrate and [γ -³²P]-ATP. The reaction mixture was incubated at 30°C for 20 minutes, resolved in SDS-PAGE, and band intensities were visualized by phosphorimaging (Packard).

Detection of mRNA

HUVECs were stimulated with ANP for 10 minutes up to 6 hours. RNA was prepared using RNeasy RNA isolation kit (Qiagen). RT-PCR experiments were performed with primers for MKP-1 (sense 5'-GCTGTGCAGCAACAGTC-3'; antisense 5'-TACCTTATGAGGACTAATCG-3') and GAPDH followed by gel electrophoresis, ethidium bromide staining, and densitometric analysis.

Antisense Experiments

HUVECs were transiently transfected with antisense (5'-cc-CACTTCCATGACCA-tgg-3') or sense (5'-cc-ATGGTCATGGAAGT-ggg-3') phosphorothioate-modified oligonucleotides as described under Mosmann³³ (phosphorothioate modifications are shown by small letters). Oligonucleotides were used in a final concentration of 0.03 μ g/well (12-well plates). The cells were transfected using an Effectene

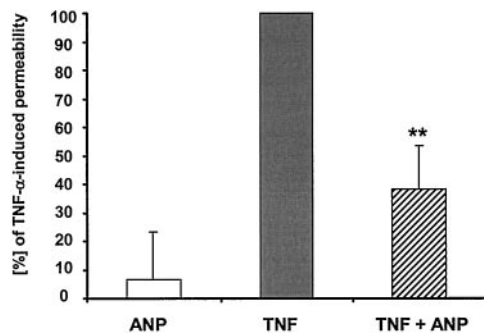


Figure 1. Permeability. HUVECs in transwell chambers were either left untreated or treated with TNF- α (25 ng/mL) alone or in combination with ANP (10^{-6} mol/L) for 24 hours, whereby ANP was added to the cells 30 minutes before TNF- α . Permeability of FITC-labeled BSA was determined as described in Materials and Methods. Values were normalized on albumin flux in untreated cells, and values of TNF- α -induced permeability increase were set as 100%. Data show mean \pm SEM of 3 different experiments prepared in triplicates or quadruplets. ** $P < 0.01$ represents significant difference from values in TNF- α -activated cells.

kit (Qiagen), whereby the transfection complex was then given to the cells for 3 hours. After addition of fresh medium, cells were either left untreated or stimulated with TNF- α in the presence or absence of ANP, which was added to the cells 30 minutes before TNF- α .

Statistical Analysis

Unless stated otherwise all experiments were done from cells of at least 3 different cell preparations. Each experiment was performed at least in triplicate. Data are expressed as mean \pm SEM. Values with $P \leq 0.05$ were considered statistically different compared with 100% or 1-fold (TNF- α -treated cells, 1 sample t test). Statistical analysis was performed with Graph Pad Prism (version 3.02).

Results

ANP Inhibits TNF- α -Induced Increase in HUVEC Permeability

Treatment of HUVECs with TNF- α leads to an increase in endothelial permeability as indicated by an increased FITC-albumin flux through endothelial monolayers (Figure 1). ANP significantly abrogated the TNF- α -induced permeability increase, whereas ANP alone showed no effect on albumin flux (Figure 1).

ANP Abrogates TNF- α -Induced Changes in Morphology and Formation of Stress Fibers

Untreated HUVECs were well-spread and showed a typical "cobblestone" morphology. On treatment of cells with TNF- α , cells started to retract, elongate, and form intercellular gaps. ANP abrogated these changes in morphology (Figure 2A).

TNF- α treatment induced formation of stress fibers and contraction of stress fibers into dense microfilamentous masses. F-actin visualized by rhodamine-phalloidin in quiescent HUVECs was concentrated in fine F-actin filaments transversing the cells (Figure 2B). TNF- α induced changes in F-actin organization, whereby the cells showed an increase and thickening of actin bundles and the formation of stress fibers. Intercellular gaps and cell retraction were indicated by large unstained areas of the image. Cotreatment of the cells

with ANP inhibited TNF- α -induced alterations in actin distribution. ANP alone had no effect on HUVEC morphology or stress fiber formation (data not shown).

Effect of ANP on Actin Polymerization

The polymerization of G-actin into F-actin was determined by recording cellular F-actin content by measuring the fluorescence in cells stained with rhodamine-phalloidin. TNF- α lead to a strong increase of F-actin content (Figure 3A). Treatment of the cells with ANP (10^{-8} to 10^{-6} mol/L) significantly inhibited TNF- α -induced actin polymerization. ANP alone did not significantly affect F-actin content.

The second messenger analogue 8-Br-cGMP partially mimicked the effect of ANP on TNF- α -induced increase in F-actin content (Figure 3), whereas 8-Br-cGMP alone did not alter the amount of F-actin (data not shown). In order to check whether ANP-induced changes in F-actin content were related to different expression of actin on treatment with ANP, whole actin content was determined by G-actin Western blots. Denaturing conditions of SDS page electrophoresis lead to depolymerization of F-actin into G-actin and therefore allow the determination of whole cellular actin content. The amount of actin was not different in cells treated with TNF- α +ANP compared with cells treated with TNF- α (Figure 3, Western blot inset).

Inhibition of HSP27 Phosphorylation

Phosphorylation of HSP27 has previously been shown to regulate actin polymerization⁷ and to contribute to stress fiber formation in endothelial cells.³⁴ In order to determine whether ANP influences actin polymerization via this pathway, phosphorylation of HSP27 was determined by the use of a phosphospecific antibody against HSP27. Treatment of the cells with TNF- α lead to a marked phosphorylation of HSP27 (Figure 4), whereas ANP significantly inhibited TNF- α -induced phosphorylation of HSP27. Changes in phosphospecific HSP27 were not due to different amounts of HSP27 as was shown by Western blot using an antibody against HSP27 (Figure 4).

p38 MAPK Activity

p38 MAPK is known to be an upstream kinase of HSP27⁹ and its role for actin polymerization has been shown in oxidant-stress-induced stress fiber formation.³⁴ In order to investigate a role for p38 MAPK in TNF- α -induced actin polymerization the effect of the p38 MAPK inhibitor SB203580 was investigated. SB203580 did not affect basal F-actin content, whereas it significantly reduced TNF- α -induced F-actin formation (Figure 5A). TNF- α -induced formation of stress fibers was also abrogated by SB203580 (actin staining, Figure 5B). The causal role for p38 MAPK activation in the TNF- α -mediated increase of permeability was assessed by employing SB203580, which in fact abrogated TNF- α -induced elevated FITC-BSA flux (Figure 5C). SB203580 alone did not affect endothelial permeability.

Because of the causal role of TNF- α -induced p38 MAPK activation for actin polymerization, an effect of ANP on p38 MAPK was investigated. MAPK activation can be measured by immunoblotting with epitope-specific anti-phosphotyrosine

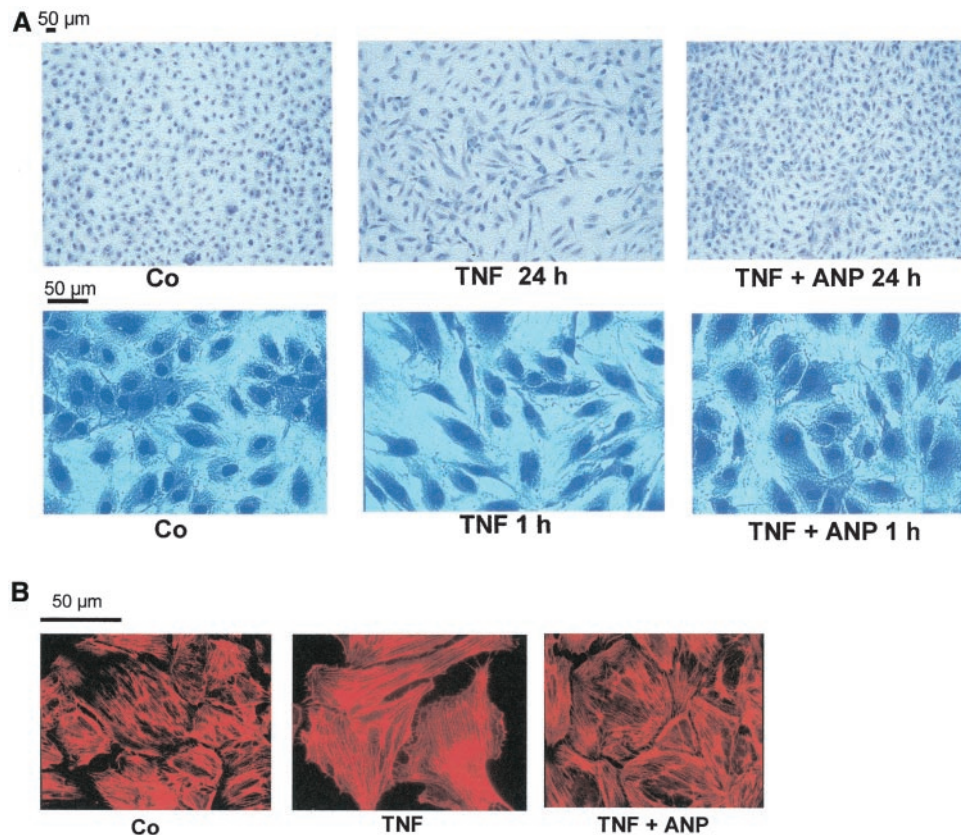


Figure 2. Morphology and cytoskeleton. A, ANP inhibits TNF- α -induced morphological alterations in HUVECs. Confluent HUVECs were cultured in either medium (Co) or in medium containing TNF- α (10 ng/mL) in the presence or absence of ANP (10^{-6} mol/L) for 1 or 24 hours (pretreatment with ANP for 30 minutes). Cells were stained as described in Materials and Methods. Pictures show representative photographs out of 4 independent experiments (Top, original magnification, $\times 100$; Bottom, $\times 200$). B, ANP attenuates TNF- α -induced formation of stress fibers. HUVECs were either untreated (Co) or activated with TNF- α (10 ng/mL) with or without ANP (10^{-6} mol/L) for 30 minutes. Cells were permeabilized and F-actin was stained with rhodamine-phalloidin. Data show 1 representative out of 3 independent experiments (original magnification, $\times 400$).

antibodies to determine MAPK phosphorylation. Alternatively, MAPK activity can be assessed by quantifying phosphorylation of specific substrates. It is possible that MAPK phosphorylation is not a fully quantitative indicator of MAPK activity, whereas enzyme substrates may not be fully specific for a certain MAPK. Therefore, both techniques for demonstrating activation of p38 were employed here. ANP significantly reduced TNF- α -mediated activation of p38 as shown by a phosphospecific p38 MAPK antibody and Western blot (Figure 5D). ANP did not affect amount of total p38 MAPK. Inhibition of p38 MAPK was dose-dependent as shown by Western blots and in vitro kinase assay (Figure 5E).

ANP Induces MKP-1 Protein and mRNA Expression

To determine the mechanism by which ANP reduces TNF- α -induced p38 MAPK activation, a potential effect of ANP on the kinase upstream of p38 MAPK, MKK3/6,⁹ was investigated. ANP did not alter TNF- α -induced activation of MKK3/6 (see online Figure 1 in the online data supplement available at <http://www.circresaha.org>).

p38 MAPK is inactivated by MAP kinase phosphatase-1 (MKP-1).⁹ We therefore determined a potential effect of ANP on MKP-1 expression. ANP significantly increased MKP-1

protein as early as 30 minutes, whereas TNF- α had no effect on MKP-1 expression (Figure 6). MKP-1 induction seems to be regulated on the transcriptional level because ANP significantly elevated MKP-1 mRNA expression (Figure 7).

Involvement of MKP-1 Induction in p38 Inhibition

We investigated a functional role of ANP-stimulated MKP-1 expression by transfecting HUVECs with either MKP-1 antisense or sense phosphorothioate-modified oligonucleotides. In order to show the functionality of our antisense strategy, we examined MKP-1 induction after antisense treatment. In fact, transfection with MKP-1 antisense, but not sense, oligonucleotides abolished ANP-induced MKP-1 expression (Figure 8A). As shown in Figure 8B, transfection with MKP-1 antisense, but not sense, oligonucleotides abrogated the inhibitory effect of ANP on p38 activation. Neither sense nor antisense oligonucleotides influenced total p38 protein levels (Figure 8B and online Figure 2). These data provided a causal relationship between ANP-mediated induction of MKP-1 and inhibition of p38 MAPK by ANP.

Discussion

TNF- α is known as a crucial mediator in the pathogenesis of atherosclerosis and thereby also represents a crucial risk

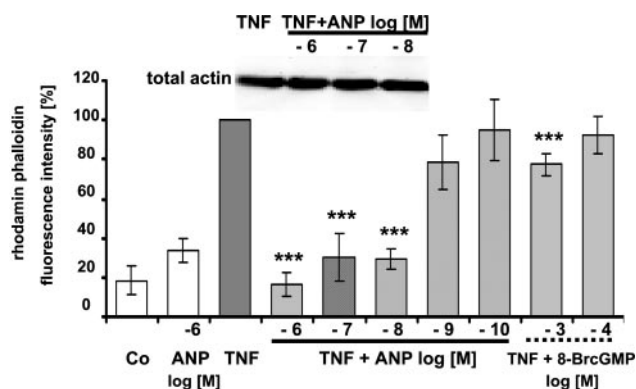


Figure 3. Actin polymerization. Cells were either left untreated (Co) or treated with TNF- α (10 ng/mL) for 1 hour. ANP (10^{-10} to 10^{-6} mol/L) or 8-Br-cGMP (10^{-3} to 10^{-4} mol/L) was added to the cells alone or 30 minutes before TNF- α . F-actin content was quantified by staining with rhodamine-phalloidin followed by fluorescence photometry. Data are expressed as percentage with 100% representing values for TNF- α treatment only. Data show mean \pm SEM of 3 to 5 independent experiments, each performed in triplicate. *** $P < 0.001$ represents significant differences compared with the values of TNF- α -activated cells. Inset, Western blot showing total actin of cells treated with TNF- α (10 ng/mL) for 1 hour with or without 30 minutes ANP pretreatment (10^{-8} to 10^{-6} mol/L).

factor in hypertension.^{1,2,4} Very recent works suggest TNF- α also as a risk factor in obesity³⁵ and obesity-associated hypertension.³⁶ In contrast, ANP has been suggested to play a protective action in the respective pathophysiology. In this context, it was described that ANP represents a lipid-mobilizing compound,³⁷ that ANP is downregulated in obesity,³⁸ and that ANP gene variants might protect against the development and progression of kidney damage in hypertension.³⁹ Molecular mechanisms of the detrimental action of TNF- α as well as the protective action of ANP in cardiovascular diseases are not completely understood. Moreover, studies on a potential interaction of TNF- α and ANP in the cardiovascular system are completely lacking. Our data demonstrate, for the first time, mechanisms by which the cardiovascular hormone ANP influences TNF- α -induced changes in endothelial cells. In HUVECs, ANP is shown to abrogate (1) the TNF- α -induced increase in permeability and alterations in HUVEC morphology and stress fiber formation. In this context, (2) evidence is provided that this effect is mediated via the NPR-A and cGMP, and (3) that the effect is connected to an inhibition of p38 MAPK, which is mediated by an induction of MKP-1.

ANP Reduces TNF- α -Induced Changes in Endothelial Morphology, Cytoskeleton, and Function

Our work provides evidence that the cardiovascular hormone ANP can abrogate changes in endothelial morphology and function. A TNF- α -induced increase in actin stress fibers followed by the formation of intercellular gaps has been reported to occur in endothelial cells.^{4,5} These changes in cytoskeletal structure are associated with an increased endothelial permeability.^{2,3} Such passage of macromolecules through the endothelium is increased at sites of inflammation

and in human arteriosclerotic plaques.² The ability of ANP to abrogate the TNF- α -induced increase in permeability is of special interest because these data suggest an antiinflammatory and antiatherogenic action for the cardiovascular hormone. This hypothesis is supported by an article by Murohara et al⁴⁰ showing a protective effect of ANP against lysophosphatidylcholine-induced endothelial dysfunction. Moreover, a cGMP-dependent inhibition of thrombin⁴¹ and oxidant⁴² induced increase in permeability has been described in bovine aortic endothelial cells. However, our study substantially extends this information because up to now nothing was known about the mechanisms underlying the protective action of ANP on TNF- α -exposed endothelium.

ANP Inhibits TNF- α -Induced Actin Polymerization Involving cGMP

The knowledge that increased permeability is closely associated with increased formation of F-actin³⁻⁶ lead us to focus on effects of ANP on actin polymerization. Little information exists on effects of ANP on stress fiber formation. Only a single study, by Sharma et al,⁴³ demonstrated that incubation of glomerular epithelial cells with ANP resulted in an apparent disassembly of stress fibers mediated via cGMP. Our work showed no effect of ANP on basal F-actin content in endothelial cells, whereas TNF- α -induced formation of F-actin was significantly reduced by ANP. The effect was shown to involve cGMP-dependent pathways because the NPR-A second messenger analogue 8-Br-cGMP partially mimicked the effect of ANP.

ANP Inhibits p38 MAPK Activation via Induction of MKP-1

Importantly, our data provide substantial information on the signaling mechanisms underlying the ANP-mediated effects on cytoskeletal actin organization. HSP27 has been closely associated with the regulation of actin polymerization,^{7,8} which represents a key step in the formation of stress fibers.⁴

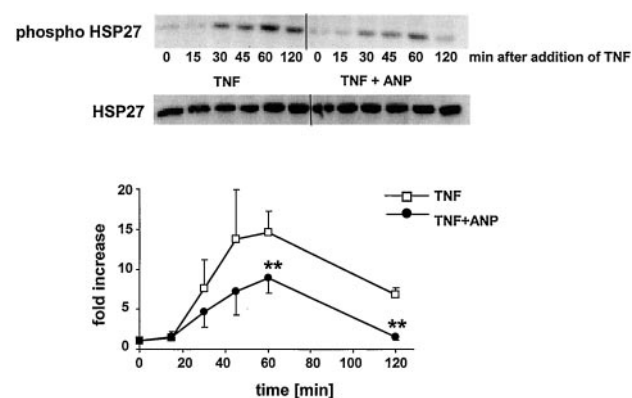


Figure 4. Phosphorylation of HSP27. HUVECs were treated with TNF- α (10 ng/mL) alone or in combination with ANP (10^{-6} mol/L) for the indicated times. Cells were harvested and Western blots with antibodies against phosphorylated HSP27 (top) or total HSP27 (middle) were performed. One representative out of 3 independent experiments is shown, each. Bottom, Densitometric evaluation expressed as x-fold increase vs 0 minutes. ** $P < 0.01$ represents significant differences compared with point 0 minutes.

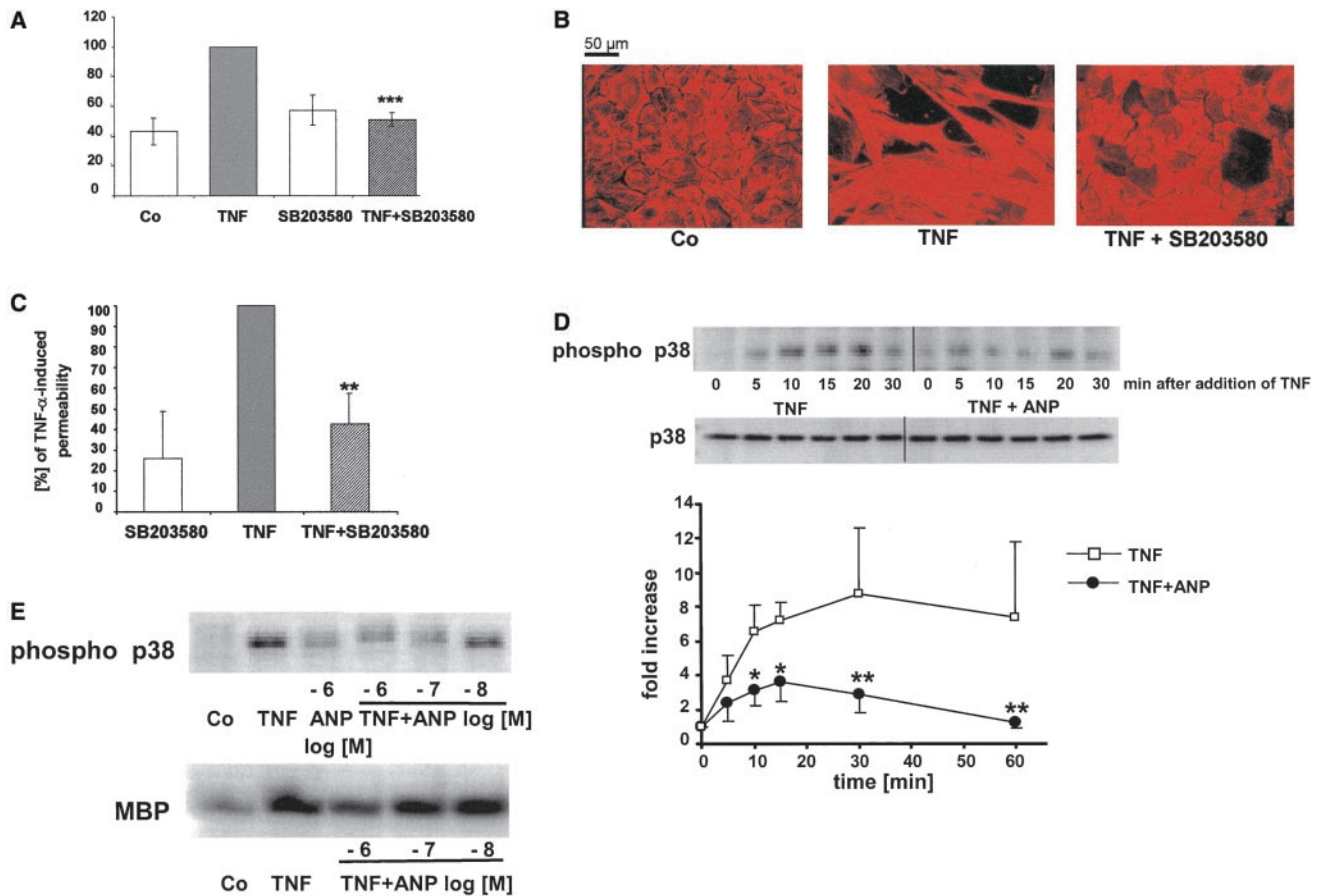


Figure 5. p38 MAPK and actin polymerization/stress fibers. **A**, SB203580 inhibits TNF- α -induced actin polymerization. HUVECs were cultured in either medium alone (Co), in medium with SB203580 (10^{-5} mol/L), or in medium containing TNF- α (10 ng/mL) in the presence or absence of SB203580 (10^{-5} mol/L). F-actin was quantified by fluorescence photometry. Values for TNF- α treatment only were referred to as 100%. Data show mean \pm SEM of 4 independent experiments, each performed in triplicate. *** $P < 0.001$ represents significant differences compared with the values for TNF- α -activated cells. **B**, SB203580 abrogates TNF- α -induced stress fibers. Cells were either left untreated (Co) or treated with TNF- α (10 ng/mL) in the presence or absence of SB203580 (5×10^{-6} mol/L) for 30 minutes. Cells were permeabilized and stained with rhodamine-phalloidin. Data show 1 representative out of 4 independent experiments (original magnification, $\times 200$). **C**, p38 MAPK and endothelial permeability. HUVECs in transwell chambers were either left untreated or treated with TNF- α (25 ng/mL) alone or in combination with SB203580 (5×10^{-6} mol/L) for 24 hours. Permeability of FITC-labeled BSA was determined as described in Materials and Methods. Values were normalized to albumin flux in untreated cells and values of TNF- α -induced permeability increase were set as 100%. Data show mean \pm SEM of 3 different experiments prepared in triplicate or quadruplet. ** $P < 0.01$ represents significant difference from values in TNF- α -activated cells. **D**, ANP inhibits TNF- α -induced phosphorylation of p38 MAPK. HUVECs were treated with TNF- α (10 ng/mL) in the presence or absence of ANP (10^{-6} mol/L) for the indicated times, whereas ANP was added to the cells 30 minutes before TNF- α . Western blots were performed using antibodies against phosphorylated and nonphosphorylated p38. Top, One representative out of 3 independent experiments. Bottom, Densitometric evaluation of signal intensities of 3 experiments, whereby * $P < 0.05$ and ** $P < 0.01$ represent significant differences compared with time point 0 minutes. **E**, Dose-dependent inhibition of TNF- α -induced p38 MAPK activity by ANP. HUVECs were either untreated (Co) or treated with TNF- α (10 ng/mL) in the presence or absence of ANP (10^{-8} to 10^{-6} mol/L), which was added to the cells 30 minutes before TNF- α . p38 MAPK activation was either shown by demonstrating phosphorylated p38 (Western blot, top, 15 minutes) or by in vitro phosphorylation assay (bottom, 10 minutes) using MBP as a substrate. Data show 1 representative out of 3 (in vitro phosphorylation) to 5 (Western blot) independent experiments, each.

We for the first time report that ANP inhibits phosphorylation of this actin capping protein. An inhibition of p38 MAPK by ANP, the kinase upstream of HSP27 phosphorylation,⁹ was shown to be responsible for this action of ANP. The observation of a decreased activation of p38 MAPK by ANP has been described in the literature before: the inhibitory action of ANP on TNF- α production was demonstrated to be mediated via an attenuation of LPS-induced activation of p38 MAPK.¹⁴ Moreover, ANP reduces VEGF-induced activation of p38 MAPK also in endothelial cells.⁴⁴ These studies support our observation

of ANP as an inhibitor of p38 MAPK. However, no information existed on the ANP-induced signaling leading to suppressed p38 activity. Our data indicate that ANP does not affect the activation of MKK3/6 upstream of p38 MAPK, but induces MKP-1¹⁰ in as short as 30 minutes via an elevation of MKP-1 mRNA. ANP is able to induce MKP-1 in mesangial cells⁴⁵ and renal tubular cells⁴⁶ as well. However, as to whether induction of MKP-1 by ANP results in inhibition of p38 MAPK, as suggested by our experiments, has not been addressed in these investigations on kidney cells. Our data clearly show a causal role of

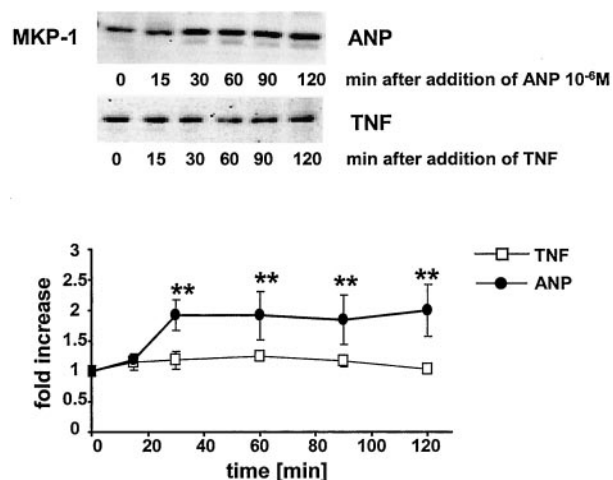


Figure 6. Induction of MKP-1. Cells were treated with ANP (10⁻⁶ mol/L) or TNF- α (10 ng/mL) for the indicated times. MKP-1 expression was investigated by Western blot. The blots show 1 representative out of 4 independent experiments, each. Bottom, Densitometric evaluation whereby $^{**}P<0.01$ represents significant differences compared with time point 0 minutes of the respective treatment.

MKP-1 in ANP-mediated p38 inhibition because MKP-1 antisense but not sense oligonucleotides abrogated the inhibitory effect of ANP on p38 MAPK activation.

Causal Relationship Between the MAPK Pathway, F-Actin Polymerization, and Macromolecule Permeability

The observation that SB203580 abrogates both F-actin polymerization as well as TNF- α -induced elevation of macromolecule permeability causally connects TNF- α -induced alterations in HUVECs with the p38 MAPK signaling cascade. Moreover and importantly, we presently show that ANP counteracts the TNF- α -induced formation of stress fibers and increased permeability by inhibiting p38 MAPK signaling.

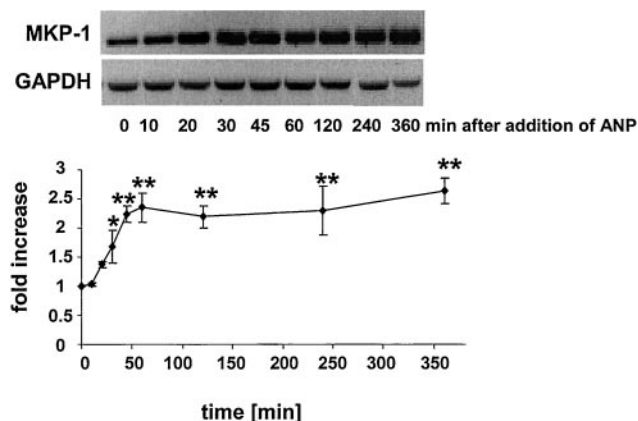


Figure 7. Induction of MKP-1 mRNA. HUVECs were treated with ANP (10⁻⁶ mol/L) for the indicated times. MKP-1 mRNA expression was investigated by RT-PCR. The blot shows 1 representative out of 3 independent experiments from different cell preparations. Bottom, Densitometric evaluation of signal intensities of 3 experiments normalized on GAPDH whereby $^{*}P<0.05$ and $^{**}P<0.01$ represent significant differences compared with the values seen at time point 0 minutes.

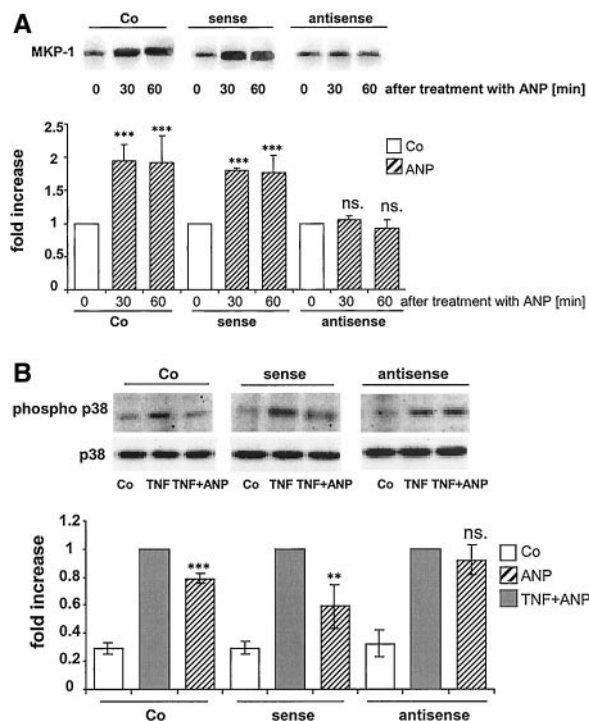


Figure 8. MKP-1 antisense. HUVECs were transfected with anti-sense or sense MKP-1 oligonucleotides for 3 hours or treated with transfection reagent without addition of DNA for the respective time (Co). After addition of fresh medium, cells were either left untreated or stimulated with ANP (10⁻⁶ mol/L; 30 or 60 minutes; A) or TNF- α (10 ng/mL; 15 minutes) in the presence or absence of ANP (10⁻⁶ mol/L; B), which was added to the cells 30 minutes before TNF- α . Western blot experiments were performed for MKP-1 or phospho p38 or total p38. A, Data show 1 representative out of 4 independent experiments. B, Western blots show 1 representative out of 4 independent experiments. Histograms show densitometric evaluation of the respective blots and represent mean \pm SEM of 4 independent experiments whereby values of TNF- α treatment were set as 100%. $^{**}P<0.001$ and $^{***}P<0.0001$ represent significant difference from values in TNF- α -activated cells.

In summary, our data provide evidence that ANP is able to preserve the structure and function of endothelial cells. These observations are of special importance because there is clear evidence that endothelial cell dysfunction is the cause of many acute and chronic vascular diseases.⁴⁷ The potency of an endogenous compound, ie, ANP, to protect against such endothelial dysfunction points to an antiinflammatory and antiatherogenic potential of this cardiovascular hormone.

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