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cGMP-Mediated Inhibition of TNF-α Production by the Atrial Natriuretic Peptide in Murine Macrophages

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The atrial natriuretic peptide (ANP) is suggested to regulate inflammatory response by alteration of macrophage functions. The aim of this study was to investigate whether ANP influences production of TNF-α. TNF-α production in murine bone marrow-derived macrophages was induced by LPS, and TNF-α secretion (±ANP) was determined by L929 bioassay. ANP dose dependently (10^-8-10^-6 M) inhibited TNF-α release by up to 95%. The effect was mediated via the guanylate cyclase-coupled A receptor, as was shown by employing dibutyryl-cGMP, the cGMP-inhibitory compound Ly-83583, and the A receptor antagonist HS-142-1. A specific ligand of the natriuretic peptide “clearance” receptor inhibited TNF-α-receptor, as was shown by employing dibutyryl-cGMP, the cGMP-inhibitory compound Ly-83583, and the A receptor antagonist HS-142-1. A specific ligand of the natriuretic peptide “clearance” receptor inhibited TNF-α production only at 10^-7 and 10^-8 M, but not at 10^-6 M. The B receptor ligand C-type natriuretic peptide showed no TNF-α-inhibitory effect. To investigate the underlying mechanism of ANP-mediated TNF-α inhibition, Northern blot was performed. ANP-treated macrophages displayed decreased TNF-α-mRNA levels. Besides the known inhibition of NF-κB activation, this study demonstrated that ANP also attenuates the activation of the proinflammatory transcription factor AP-1 (gel shift assay). ANP did not alter subunit composition of AP-1 complexes, as was shown by supershift assays applying anti-c-jun and anti-c-fos Abs. To get information on the ANP effect for human inflammatory processes, we investigated cytokine production in human LPS-activated blood. ANP significantly attenuated production of TNF-α and IL-1β without affecting production of IL-10 and IL-1ra. In summary, ANP was shown to attenuate TNF-α production of LPS-activated macrophages via cGMP. The inhibition is suggested to involve transcriptional processes that are the result of reduced activation of responsible transcription factors. The Journal of Immunology, 2000, 165: 175–181.

The 28-aa atrial natriuretic peptide (ANP) is a cardiovascular hormone mainly secreted by heart atria (1, 2). Most investigations deal with the diuretic, natriuretic, and vasodilating action of ANP regarding the regulation of volume-pressure homeostasis (for review, see Refs. 1–3). Most of the effects of ANP in this regard are mediated by the guanylate cyclase-coupled A receptor (NPR-A) (4). The C-type natriuretic peptide (CNP) is the specific ligand for the B receptor (NPR-B) (5). CNP is suggested to be the major natriuretic peptide (NP) in the brain (6), but it was also found in peripheral cells such as endothelial cells (7) and macrophages (8, 9). Compared with ANP, CNP has insignificant natriuretic and diuretic, but distinct vasoactive properties (6, 7). Thus, CNP may play a physiological role quite different from that of ANP. All NP bind to the non-guanylate cyclase-linked C-receptor (NPR-C), which is known for its clearance function (5). An increasing amount of data report that several in vitro effects of ANP are mediated by an NPR-C-initiated inhibition of adenylate cyclase via an interaction with G-proteins (5, 10).

The functions of the NP are not restricted to the regulation of volume homeostasis. ANP and its receptors were demonstrated to be expressed in diverse tissues besides the cardiovascular and renal system (11). Our previous work drew attention to a new aspect in the biological profile of ANP, i.e., its interference with the immune system (12). ANP and its receptors were shown to be expressed in thymus (13, 14) as well as in macrophages (8, 9, 15). Concerning functional consequences, ANP was demonstrated to inhibit thymocyte proliferation (14) and thymopoiesis (16). Furthermore, ANP stimulates phagocytosis and production of reactive oxygen species in macrophages (17) and inhibits inducible NO synthase (iNOS) in LPS-activated macrophages (15, 18). This effect was demonstrated to be mediated via a destabilization of iNOS mRNA and by reduced activation of NF-κB initiated by ANP (19). ANP was also shown to attenuate ischemia-reperfusion injury of the liver via its guanylate cyclase-linked A receptor (20). Ischemia-reperfusion injury is considered as a state of inflammation due to the activation of macrophages and the secretion of TNF-α (21). Interestingly, concerning the mechanism underlying the protective effect exerted by ANP on this kind of cell damage, the inhibition of NF-κB is suggested to be involved (20). The knowledge about the influence of ANP on immune functions led us to hypothesize that ANP influences LPS-induced TNF-α production in macrophages. This central proinflammatory cytokine is regulated transcriptionally, whereby the two transcription factors NF-κB and AP-1 are involved (22). AP-1 is a family of related transcription factors, which frequently consist of either c-jun/c-fos heterodimers or c-jun/c-jun homodimers (23). Changes in AP-1 activity in response to extracellular signals are regulated both on the level of transcription of jun and fos genes and by posttranslational modification of preexisting AP-1 (c-jun/c-jun). The NF-κB complex in its inactive form is located in the cytosol and consists of two DNA binding subunits, p50 and p65, which are associated with an inhibitory protein, I-κB (inhibitory protein that dissociates from
NF-κB). Different stimuli, such as bacterial LPS, lead to a phosphorylation and degradation of I-κB upon which NF-κB can translocate into the nucleus. There, NF-κB binds to specific promoter sequences of proinflammatory genes, such as TNF-α, and initiates their transcription (24).

The macrophage cytokine TNF-α, together with other inflammatory mediators (25), plays a key role in many pathophysiological conditions, such as rheumatoid arthritis (26), atherosclerosis (27), or septic shock (28). ANP was previously shown to be an autocrine regulator of iNOS (19), an enzyme strongly induced in inflammatory processes (29) such as septic shock (30). The observation that ANP mediates macrophage activation is particularly interesting because ANP concentrations are highly elevated in septic shock (31); moreover, LPS-exposed macrophages were shown to produce increased ANP (9). Thus, due to its effects on key events of macrophage activation, such as the activation of NF-κB, ANP may represent a promising autocrine substance modulating TNF-α production. Therefore, the aims of this study were 1) to investigate whether ANP influences the production of this central proinflammatory cytokine, 2) to consequently determine the type of receptor whether ANP stimulates or inhibits TNF-α production, and 3) to elucidate whether a potential ANP effect, 3) to elucidate whether the C-type NP inhibits TNF-α production, and 4) to obtain information on the underlying mechanism of ANP-mediated TNF-α inhibition.

Materials and Methods

**Materials**

Rat ANP 99–126 was purchased from Calbiochem-Novabiochem (Bad Soden, Germany), and CNP and CAF were purchased from Saxon Biochemicals (Hannover, Germany). HS-142-1 was a gift from Dr. Y. Matsuda (Tokyo Research Laboratories, Tokyo, Japan). TNF-α cDNA was obtained from Dr. K. Decker (University of Freiburg, Freiburg, Germany). The antiserum against the macrophage Ag F4/80 was from Serotec (Wiesbaden, Germany); cell culture medium (RPMI 1640), FCS, penicillin/streptomycin, and TRIzol were from Life Technologies (Karlruhe, Germany) and Biochrom (Berlin, Germany); [α-32P]UTP (800 Ci/mmol) and [γ-32P]ATP (3000 Ci/mmole) were from Amersham (Braunschweig, Germany); dexamethasone solution was ordered from Centravet (Bad Bentheim, Germany); and AP-1 and AP-2 binding oligonucleotides, SP6 polymerase, and T4 polynucleotide kinase were obtained from Boehringer Ingelheim Bioproducts (Heidelberg, Germany). Bradford protein assay was from BioRad (Munich, Germany). All other materials were purchased from either Sigma (Deisenhofen, Germany) or ICN Biomedicals (Eschwege, Germany).

**Cell culture**

Mouse bone marrow macrophages (BMM) were prepared as described previously (8), seeded at a density of 2 × 10⁵ cells/ml in 24-well tissue plates, and grown for 5 days (5% CO₂, 37°C) in RPMI 1640 medium supplemented with 20% L-929 cell-conditioned medium, 10% heat-inactivated FCS, and penicillin (100 U/ml)/streptomycin (100 µg/ml). L-929 cell-conditioned medium was removed at least 12 h before experiments. BMM were grown in 24-well plates and stimulated with LPS (1 µg/ml) or dexamethasone (10⁻⁶ M), or in a combination of LPS (1 µg/ml) and dexamethasone (10⁻⁶ M). Culture supernatants were assayed for TNF-α production using a bioassay (see Materials and Methods). Data are expressed as percentage of TNF-α concentration accumulated in the supernatant of LPS-activated macrophages (100%) and represent the mean ± SEM of three (dexamethasone, ANP 10⁻⁶ M) to 12 independent experiments performed in triplicate. ***p < 0.0001; significant difference compared with the values seen in LPS-activated cells (one sample t-test).

**Detection of TNF-α mRNA**

BMM were stimulated with or without LPS (1 µg/ml) in the presence or absence of ANP (10⁻⁶ M) or dexamethasone (10⁻⁶ M) for 4 h (24-well plates). RNA was prepared using TRIzol reagent and was pooled from six wells. Northern blot analysis with total RNA (15 µg) was performed as described previously (8). Membranes were hybridized to 32P-labeled cRNA probes (2 × 10⁶ cpm/ml). The cDNA probe was a Smal linearized rat TNF-α cDNA fragment subcloned in a pSPT18 vector (from Dr. Decker). The TNF-α probe was labeled with [α-32P]UTP (50 µCi, Amersham) and Sp6 RNA polymerase. Signal intensities were evaluated by densitometric analysis (EASY plus system; Herolab, Wiesloch, Germany). To control for the amounts of intact mRNA, membranes were rehybridized with a 32P-labeled β-actin probe (2 × 10⁶ cpm/ml) described in Ref. 34. Signal intensities were evaluated by densitometry, whereby signals for TNF-α were corrected by β-actin signals. Ratios of LPS-treated cells were set 100%.

**Preparation of nuclear extracts**

BMM were grown in 24-well plates and stimulated with LPS (1 µg/ml) in the presence or absence of ANP (10⁻⁶ M) for 30 min. Nuclear extracts were prepared as described in Ref. 35. Briefly, cells were washed with PBS, resuspended in 400 µl hypotonic buffer A (10 mM HEPEs [pH 7.9),
FIGURE 2. Characterization of the NPR responsible for the ANP-induced TNF-α-inhibition in BMM. Cells were treated with LPS (1 μg/ml; 4 h), and TNF-α production was measured (bioassay) and referred to as 100%. A, Effect of dibutyryl-GMP (dibut) at $10^{-3}$–$10^{-5}$ M added simultaneously with LPS. Results are expressed as percentage of TNF-α accumulation in the supernatant of LPS-treated cells and represent the means ± SEM of three (10$^{-4}$ and 10$^{-5}$ M) and twelve (10$^{-3}$ M) independent experiments performed in triplicate. B, Effect of the NPR-A antagonist HS-142-1 (10 and 100 μg/ml) with and without ANP (10$^{-6}$ M) added simultaneously with LPS. Means ± SEM of three independent experiments performed in triplicate. C, Effect of coincubation of ANP (10$^{-6}$ M) and Ly-83583 (10$^{-7}$–10$^{-9}$ M), an antagonist of cGMP generation, added simultaneously with LPS. Means ± SEM of three independent experiments performed in triplicate. D, Effect of the NPR-C-specific ligand cANP (10$^{-6}$–10$^{-8}$ M). Means ± SEM of four (10$^{-4}$ and 10$^{-5}$ M) and seven (10$^{-6}$ M) independent experiments performed in triplicate. ***, p < 0.0001, significant difference compared with the values seen in LPS-activated cells (one sample t test); +, p < 0.0001, significant difference compared with the values seen in LPS-activated cells (one sample t test); *, p < 0.05, significant difference compared with LPS + ANP treatment (unpaired t test); **, p < 0.01, significant difference compared with LPS + ANP treatment (unpaired t test).

EMSA

A 22-mer double-stranded oligonucleotide probe containing a consensus binding sequence for AP-1 (5’-CGG TTG ATG AGT CAG CGG GAA-3’) was 5’ end-labeled with [α-32P]ATP (10 μCi) using T4 polynucleotide kinase. Ten microliters of nuclear protein was incubated (20 min at room temperature) in a 15-μl reaction volume containing 10 mM Tris-HCl (pH 7.5), 5 × 10$^{-6}$ cmol radiolabeled oligonucleotide probe, 2 μg poly(dIdC), 4% glycerol, 1 mM MgCl$_2$, 0.5 mM EDTA, 50 mM NaCl, and 0.5 mM DTT. Nucleoprotein-oligonucleotide complexes were resolved by electrophoresis (4.5% nondenaturing polyacrylamide gel; 100 V). The gel was autoradiographed with an intensifying screen at −70°C overnight. Specificity of the DNA-protein complex was confirmed by competition with a 100-fold excess of unlabeled AP-1 and AP-2 (5’-GAT CGA ACT GAC CGC CGG CCC GT-3’) binding sequences, respectively. For supershift analysis, 1 μg of Abs against c-Jun or c-Fos (Santa Cruz Biotechnology, Heidelberg, Germany) was added to the reaction mixtures 10 min before the addition of radiolabeled probe. Preimmune rabbit IgG served as a control.

Cytokine secretion of human whole blood

Cytokine secretion from human whole blood was determined as described previously (37). Briefly, heparinized blood was drawn from healthy volunteers and diluted 1:5 with cell culture medium RPMI 1640 supplemented with penicillin-streptomycin. Further heparin (2 IU/ml final concentration) was added to prevent blood coagulation as a result of dilution. LPS from Salmonella abortus equi was added simultaneously in a volume of 10 μl.

After 24 h of incubation at 37°C and 5% CO$_2$, the vials were shaken gently, cells were sedimented by centrifugation, and the supernatants were frozen in aliquots at −80°C until cytokine measurement.

Cytokines were measured by sandwich ELISA. IL-1β and TNF-α Ab pairs were from Endogen (Eching, Germany), and IL-10 Ab pairs were from R&D Systems (Wiesbaden, Germany). rIL-1β (Endogen), recombinant TNF-α (Bender, Vienna, Austria), and recombinant IL-10 (R&D Systems) were used as standards. Streptavidin-conjugated peroxidase (Di-anova, Hamburg, Germany) and the chromogen tetramethylbenzidine were used for detection of the immune complexes.

**Statistical analysis**

All experiments were performed at least three times. Data are expressed ± SEM. Values with p < 0.01 were considered significantly different compared with 100% (LPS-treated cells only) by one sample t test.

**Results**

ANP inhibits TNF-α production in BMM

Murine bone marrow-derived macrophages were stimulated with LPS (1 μg/ml) for 4 h to evoke TNF-α synthesis. TNF-α in the supernatant was measured by determining the cytotoxic activity on L929 cells by employing recombinant TNF-α as a standard. Co-incubation of BMM with ANP (10$^{-8}$–10$^{-6}$ M) and LPS (1 μg/ml) resulted in a dose-dependent, significant reduction of TNF-α production up to 95% (Fig. 1). Dexamethasone, a known inhibitor of TNF-α production (38), completely abolished TNF-α secretion (Fig. 1) and served as a control. ANP (10$^{-6}$ M) in the absence of LPS did not alter the basal TNF-α secretion and did not interfere with the L929 bioassay (data not shown).
Receptor selectivity of the ANP effect on TNF-α synthesis

To determine which NP receptor mediates the inhibitory effect of ANP on TNF-α synthesis, the following experiments were performed with LPS-stimulated cells. As shown in Fig. 2A, the stable analogue of cGMP, dibutyryl-cGMP, at a concentration of 10^{-3} and 10^{-4} M significantly reduced TNF-α secretion by up to 70%. Furthermore, an antagonist of the guanylate cyclase-coupled receptor (NPR-B), CNP, partially abolished the reduction of TNF-α secretion by ANP (10^{-6} M) in BMM (Fig. 2B). Ly-83583 (10^{-7} and 10^{-6} M), a compound known to inhibit cGMP production (40), partially abolished the reduction of TNF-α secretion by ANP (10^{-6} M) (Fig. 2C). The specific NPR-C ligand cANF did not elicit a significant decrease in TNF-α secretion at a concentration of 10^{-6} M (Fig. 2D). However, cANF significantly reduced TNF-α production in concentrations of 10^{-3} and 10^{-4} M. ANP, dibutyryl-cGMP, HS-142-1, Ly-83583, and cANF were tested for their effect on TNF-α synthesis in unstimulated cells and did not exhibit any effect (data not shown).

Effect of CNP on TNF-α synthesis of BMM

BMM were previously shown to express all types of NP receptors (15) and therefore may represent targets for all types of NP. To elucidate whether the TNF-α-inhibitory effect is specific for the binding of ANP to the NPR-A, we examined the ligand for the other guanylate cyclase-coupled receptor (NPR-B), CNP. As shown in Fig. 3, CNP even at a concentration of 10^{-6} M did not affect TNF-α formation of LPS-activated BMM (Fig. 3). CNP had no effect on basal TNF-α production and did not interfere with the TNF-α bioassay (data not shown).

Effect of ANP on TNF-α synthesis of BMM

1. **Materials and Methods**

To determine the effect of ANP on TNF-α synthesis, the following experiments were performed with LPS-stimulated cells. As shown in Fig. 2A, the stable analogue of cGMP, dibutyryl-cGMP, at a concentration of 10^{-3} and 10^{-4} M significantly reduced TNF-α secretion by up to 70%. Furthermore, an antagonist of the guanylate cyclase-coupled receptor (NPR-B), CNP, partially abolished the reduction of TNF-α secretion by ANP (10^{-6} M) in BMM (Fig. 2B). Ly-83583 (10^{-7} and 10^{-6} M), a compound known to inhibit cGMP production (40), partially abolished the reduction of TNF-α secretion by ANP (10^{-6} M) (Fig. 2C). The specific NPR-C ligand cANF did not elicit a significant decrease in TNF-α secretion at a concentration of 10^{-6} M (Fig. 2D). However, cANF significantly reduced TNF-α production in concentrations of 10^{-3} and 10^{-4} M. ANP, dibutyryl-cGMP, HS-142-1, Ly-83583, and cANF were tested for their effect on TNF-α synthesis in unstimulated cells and did not exhibit any effect (data not shown).

2. **Results**

- **Northern blot analysis of TNF-α-mRNA.** Total RNA was isolated from macrophages, which were either unstimulated (Co) or treated with LPS (1 μg/ml) in the presence or absence of ANP (10^{-6} M) and dexamethasone (dexa; 10^{-5} M), respectively, for 4 h. Fifteen micrograms of total RNA was loaded per lane and hybridized to a 32P-labeled cRNA probe for TNF-α and β-actin-mRNA, respectively. A representative autoradiograph is shown in the upper panel. The lower panel shows densitometric analysis of five independent experiments. ***, p < 0.01; significant difference compared with the values seen in LPS-activated cells (one sample t test).**

3. **Binding specificity was determined by including a 100-fold excess of unlabeled AP-1 binding sequence or by adding a nonspecific oligonucleotide (AP-2) in the DNA binding reactions performed with nuclear extracts from LPS-treated BMM. The autoradiograph shows one representative of five independent experiments.**

4. **Effect of CNP on TNF-α synthesis of BMM.**

BMM were previously shown to express all types of NP receptors (15) and therefore may represent targets for all types of NP. To elucidate whether the TNF-α-inhibitory effect is specific for the binding of ANP to the NPR-A, we examined the ligand for the other guanylate cyclase-coupled receptor (NPR-B), CNP. As shown in Fig. 3, CNP even at a concentration of 10^{-6} M did not affect TNF-α formation of LPS-activated BMM (Fig. 3). CNP had no effect on basal TNF-α production and did not interfere with the TNF-α bioassay (data not shown).
ANP treatment reduces TNF-α mRNA levels

To investigate the mechanism of regulation of TNF-α production by ANP, Northern blot analysis was performed to determine whether ANP inhibits TNF-α mRNA accumulation when added simultaneously with LPS. BMM were activated with LPS (1 μg/ml) in the presence or absence of ANP (10⁻⁶ M), and mRNA was isolated. In unstimulated cells, no TNF-α mRNA was detectable (Fig. 4). ANP (10⁻⁶ M) caused a marked reduction of LPS-induced TNF-α mRNA steady-state levels. Dexamethasone (10⁻⁵ M), a known inhibitor of TNF-α induction (38), completely blocked TNF-α mRNA accumulation. Rehybridization with a β-actin probe served as a control for the amounts of intact RNA.

ANP inhibits AP-1 activity

Because ANP is known to interfere with transcriptional processes (19, 41), we determined its effect on binding activity of AP-1, a transcription factor known to be important for TNF-α induction (22). AP-1 binding activity of nuclear extracts was assessed by EMSA after stimulation of cells with LPS (1 μg/ml) for 30 min. Formation of the specific DNA probe-AP-1 complex was markedly reduced when nuclear extracts of cells coincubated with ANP (10⁻⁶ M) were employed (Fig. 5). Binding specificity was determined by addition of a 100-fold excess of unlabeled AP-1 or AP-2 binding sequence. Excess of unlabeled AP-1 binding sequence abolished binding reaction to the labeled sequence, whereas AP-2 binding sequence showed no significant influence on binding reaction (Fig. 5).

No alteration of subunit composition of AP-1 complexes by ANP

Fig. 5 shows a markedly reduced AP-1 DNA binding activity in ANP + LPS-treated cells compared with LPS treatment only. Supershift analysis employing specific Abs against the most important c-jun and c-fos subunits both resulted in markedly reduced complex formation. This observation shows that AP-1 DNA binding proteins in LPS-treated cells are predominantly composed of c-fos and c-jun (Fig. 6), although no supershifted ones were observed. No marked alteration of AP-1 composition could be detected in ANP (10⁻⁶ M) cotreated cells compared with those that received LPS treatment only (Fig. 6). Addition of preimmune IgG had no effect on binding reaction (data not shown).

ANP influences cytokine production in whole human blood

To investigate whether our shown data could be extended on other species and cell types, we determined cytokine release in LPS-activated whole human blood from healthy donors. ANP (10⁻⁷ and 10⁻⁶ M) significantly inhibited production of the proinflammatory cytokines TNF-α and IL-1β (Fig. 7). ANP showed no effect on the secretion of IL-1ra and IL-10 (Fig. 7).

Discussion

The induction of TNF-α in macrophages represents an important pathomechanism in diverse inflammatory processes (26, 27, 28). Therefore, special interest focuses on the regulatory mechanism of TNF-α production and on tools for potential pharmacological intervention (42). By characterization of a novel endogenous substance (i.e., ANP) regulating TNF-α expression, this study might therefore be of special interest. The presented work investigates for the first time mechanisms by which the cardiovascular hormone ANP selectively influences TNF-α production by macrophages.

The fact that inhibition of TNF-α production by ANP is mediated via the NPR-A was conclusively demonstrated because dibutyryl-cGMP dose dependently mimicked the ANP effect. Employment of the microbial polysaccharide HS-142-1, which selectively
blocks the guanylate cyclase-linked NP receptors and cGMP production (39), dose dependently reversed the ANP effect. Because no soluble guanylate cyclase has been detected in the macrophages employed (19), the reversal of the ANP effect by L-83583 can be attributed to an inhibition of cGMP production linked to the particular guanylate cyclase-linked NPR-A receptor. In accordance with our observations, other data report an inhibition of TNF-α production by cyclic nucleotides (43, 44, 45), both cGMP and cAMP. The effect was observed by the employment of cGMP analogues as well as of phosphodiesterases inhibitors in murine and human mononuclear cells. However, in human monocytes, ANP exerted no effect on TNF-α production (46). This observation was explained by the lack of functional NPR-A on the cells investigated and thus the missing cGMP answer after ANP treatment (46). On the other hand, Suga et al. (47) reported a significant increase in cGMP production in THelper precursor-1-derived human macrophages after ANP treatment, whereas CNP was without effect. This observation may explain our finding that CNP did not affect TNF-α secretion.

In contrast to our findings, cGMP was also reported to stimulate TNF-α synthesis (48–51). This discrepancy requires explanation: the cells in which cGMP displayed a TNF-α-inducing effect were either from rat or human source and represented different macrophage populations, such as Kupffer cells or peritoneal macrophages. These data together with our results lead to the suggestion that the effect of cGMP on TNF-α synthesis is highly dependent on species and cell type. To get a first hint toward a potential relevance of our findings during inflammatory processes in man, we performed experiments using whole human blood. As previously shown, human whole blood cytokine release offers the opportunity to assess primary leukocyte responses in a physiological cell environment without major preparation artifacts (37). Moreover, data obtained using this ex vivo (52) system has been shown to go in parallel with human in vivo data (53). Importantly, ANP in this cellular system also exerted its TNF-α inhibitory action and additionally attenuated IL-1β secretion. These interesting findings suggest an anti-inflammatory potential of ANP in humans as well.

Surprisingly, the specific NPR-C ligand cANF exhibited an inhibitory effect on TNF-α production only in lower concentrations (10⁻⁷ and 10⁻⁸ M) and not in concentrations as high as 10⁻⁶ M. This kind of effect exerted by cANF might be slightly linked to the cAMP system because activation of NPR-C is known to alter adenylate cyclase activity (10). cAMP was shown to dose dependently enhance or suppress TNF-α release (45, 50, 51). Activation of NPR-C with cANF may specifically influence cAMP levels and therefore may reveal effects that are not related to the cGMP-dependent inhibition of TNF-α release exerted by ANP.

Previous data demonstrated that macrophages express mRNA coding for all three receptor subtypes, i.e., NPR-A, NPR-B, and NPR-C (15). The fact that macrophages express the NPR-B led us to hypothesize that they should be target cells for CNP action. Interestingly, CNP was demonstrated to be highly increased in septic shock patients (54), leading to the anticipation that CNP may be involved in respective inflammatory processes, such as TNF-α production. However, the property to inhibit TNF-α secretion seems to be specific for ANP because CNP displayed no activity. This observation is in concordance with previous observations in macrophages showing a lack of effect of CNP on iNOS, whereas ANP represents a potent inhibitor of this proinflammatory enzyme (15). No difference in stability between ANP and CNP is responsible for this fact (15). Others also reported that CNP elicits very low or no biological activity in different cell systems despite the presence of NPR-B (55–57). One possible explanation for this observation might be the heterogeneity of the NPR-B receptor.

In diverse tissues, two forms of NPR-B receptor could also be detected in macrophages (15, 58). These two forms of NPR-B differ from each other by a 75-bp deletion at the third flanking region and possess practically the same high binding affinity for CNP (58). However, the shorter form could not induce cGMP production upon binding by CNP (56). The expression of both forms of NPR-B transcripts in the macrophages was shown by us previously (15). Because the ANP effect on TNF-α synthesis is shown to be mediated by cGMP, an insufficient amount of cGMP produced by CNP may indeed be responsible for the lack of TNF-α inhibition by this peptide.

After demonstrating a specific inhibitory action of ANP on TNF-α production, the molecular mechanism of action should be investigated. Because TNF-α is a transcriptionally regulated cytokine (22), the effect of ANP on TNF-α mRNA had to be determined. Northern blot analysis revealed markedly reduced levels of TNF-α mRNA initiated by ANP. The levels of TNF-α mRNA are mainly controlled via activation of respective transcription factors (22, 23). We hypothesize that ANP decreases the transcription of TNF-α mRNA by interfering with two prominent transcription factors involved in TNF-α regulation, NF-κB and AP-1 (22). In this study, we demonstrate for the first time that ANP also inhibits activation of AP-1 in macrophages. This further pivotal proinflammatory transcription factor is known to be involved in TNF-α gene expression (22, 23). Because different AP-1 complexes are suggested to exhibit different transcriptional activities (23), we investigated whether ANP alters AP-1 composition. However, a modulation of subunit composition by ANP could not be observed.

Few data exist concerning the influence of cGMP and ANP on AP-1 activation, respectively, and data are controversial. In accordance with our results, Isono et al. (59) reported that ANP inhibits activation of AP-1 in glomerular mesangial cells. In contrast, cGMP analogues were demonstrated to activate transcription from AP-1-responsive promoters in thyroid follicular cells as well as in fibroblasts (60). These data together with our results support the notion that the influence of cGMP on AP-1-dependent gene transcription might represent a highly cell type-specific and stimulus-dependent event. It is important to note that ANP has recently been shown to interfere with other cell signaling systems that control gene transcription, e.g., ANP abrogates endothelin-3-induced stimulation of egr-1 transcription and basic fibroblast factor transactivation in astrocytes (41). Furthermore, ANP was shown to attenuate activation of NF-κB in different cell/organ systems (19, 20). Taken together, increasing evidence suggests ANP as an important regulator of gene transcription.

Modulation of TNF-α synthesis by ANP may have broad implications in situations such as endotoxic shock (28), where in fact increased ANP plasma levels have been reported (31). Thus, our data lead us to suggest ANP as an endogenous regulator of inflammatory response.

In summary, we could demonstrate a novel mechanism of action for the cardiovascular hormone. ANP specifically interacts with TNF-α production of LPS-activated macrophages and whole human blood cells. This effect is mediated via cGMP and may involve transcriptional inhibition because of attenuated activation of AP-1.

Acknowledgments

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