Autocrine Regulation of Inducible Nitric-oxide Synthase in Macrophages by Atrial Natriuretic Peptide*

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Alexandra K. Kiemer and Angelika M. Vollmar‡

From the Institute of Pharmacology, Toxicology and Pharmacy, University of Munich, Königstrasse 16, 80539 Munich, Germany

Atrial natriuretic peptide (ANP), a cardiovascular hormone, has been shown to inhibit synthesis of nitric oxide in lipopolysaccharide (LPS)-activated mouse bone marrow-derived macrophages via activation of its guanylate cyclase-coupled receptor. The goal of the present study was to elucidate the potential sites of inducible nitric-oxide synthase (iNOS) regulation affected by ANP and revealed the following. 1) ANP and dibutyryl-cGMP did not inhibit catalytic iNOS activity measured by the conversion rate of L-[3H]arginine to L-[3H]citrulline in homogenates of LPS-treated cells. 2) Pretreatment of cells with ANP dose-dependently reduced the LPS-induced L-[3H]citrulline production that has been shown to be due to reduced iNOS protein levels detected by Western blot. 3) ANP does not alter the ratio of catalytically active iNOS dimer versus inactive iNOS monomer considered to be a major post-translational regulatory mechanism for the enzyme. 4) Macrophages exposed to ANP display decreased LPS-induced iNOS mRNA levels. 5) Importantly, two basic mechanisms seem to be responsible for this observation, i.e. ANP specifically induced acceleration of iNOS mRNA decay and ANP reduced binding activity of NF-κB, the transcription factor predominantly responsible for LPS-induced iNOS expression in murine macrophages. Moreover, 6) ANP acts via an autocrine mechanism since recently ANP was shown to be secreted by LPS-activated macrophages, and we demonstrated here that LPS-induced NO synthesis was increased after blocking the binding of endogenous ANP by a receptor antagonist. These observations suggest ANP as a new autocrine macrophage factor regulating NO synthesis both transcriptionally and post-transcriptionally. ANP may help to balance NO production of activated macrophages and thus may allow successful immune response without adverse effects on host cells.

Atrial natriuretic peptide (ANP) is a 28-amino acid polypeptide secreted mainly by the heart atria in response to atrial stretch. The main and best studied actions of ANP are geared toward the regulation of volume pressure homeostasis (for review see Refs. 1 and 2). There are two biochemically and functionally distinct classes of natriuretic peptide receptors (NPR). Clearance receptors (NPR-C) are by far the most abundant class of NPR. Besides their well established role in removing ANP from the circulation, the NPR-C elicit biological functions by interacting with G-proteins (2, 3). The guanylate cyclase-coupled receptors (NPR-A) are signaling receptors that mediate all known cardiovascular and renal effects of ANP via cGMP (2, 4). The functions of the natriuretic peptides, however, are not restricted to the regulation of volume homeostasis as suggested earlier by demonstration of ANP and its receptors in diverse tissues besides the cardiovascular and renal system (5). ANP was suggested to play a role in the immune system because thymus (6–8) and macrophages (9, 10) are sites of synthesis of the natriuretic peptide and its receptors. In the course of functional investigations concerning ANP in the immune system, the peptide was found to inhibit maturation and differentiation of fetal thymus (11) as well as proliferation of thymocytes of adult animals (8). Recently, ANP was shown to reduce nitrite accumulation in lipopolysaccharide (LPS)-activated murine macrophages (10). Thus, the peptide might interfere with the synthesis of a mediator that plays an important role in inflammation and host defense response (12). The enzyme nitric-oxide synthase (NOS), which catalyzes the synthesis of NO from L-arginine, exists in three different isoforms that differ in their tissue distribution, calcium dependence, and in the regulation of their expression (13, 14). The two constitutive isoforms expressed in neurons (neuronal nitric-oxide synthase; NOS I) and endothelial cells (endothelial nitric-oxide synthase; NOS III) are calcium/calmodulin-dependent. They are mainly involved in neurotransmission and vascular regulation, respectively (15). A major function of NO derived from the inducible NO synthase (iNOS; NOS II) is target cell cytotoxicity. Target cells may include tumor cells as well as bacteria, viral particles, and other microorganisms (12). However, NO produced by iNOS of macrophages (as well as other cells) also has the potential for adverse activities depending on its concentration and site of release. These include the induction of severe hypotension and cardiovascular shock and cytotoxicity toward host cells such as vascular cells, lymphocytes, or even macrophages themselves (12). Therefore, a better understanding of the physiological regulation of iNOS is important. The major activator of iNOS in macrophages has been shown to be bacterial lipopolysaccharide (LPS) (12, 16). Co-stimulatory effects were demonstrated for INF-γ and a variety of other cytokines such as TNF-α, IL-2, INF-α, and -β (12).

So far, little is known about what terminates the production of NO by macrophages. However, this is of particular impor-

natriuretic peptide receptor; PBS, phosphate-buffered saline; PDTC, pyrroclidinedithiocarbamate; PMSF, phenylmethylsulfonyl fluoride; SNP, sodium nitroprusside; TNF-α, tumor necrosis factor-α.
tance regarding the severe pathophysiologic effects of sustained NO production such as circulatory failure and tissue damage. Again cytokines, i.e. transforming growth factor-β, IL-4, and IL-10, have been described to suppress NO release of macrophages (12, 17). The underlying mechanisms appeared to be different for the respective cytokines (12, 17, 18). The observation that ANP, a circulating hormone best known for its vasodilative effects, inhibits NO synthesis in activated macrophages is particularly interesting since ANP concentrations are highly elevated in septic shock (19), and moreover LPS-exposed macrophages were shown to produce increased ANP (9). Thus, ANP may be a novel autocrine substance modulating NO production. Consecutively, aim of the present study was to clarify the basic mechanisms underlying the inhibition of NO synthesis by ANP.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mouse ANP 99-126 was purchased from Calbiochem (Bad Soden, Germany). HS-142-1 was a gift from Dr. Matsuda, Tokyo Research Laboratories, Tokyo, Japan. iNOS cDNA probe was provided by Dr. Kleinert, University of Mainz, Germany; COX-2 cDNA was a gift from Dr. Heschman, UCLA; TNF-α cDNA was obtained from Dr. Decker, University of Freiburg, Germany; IL-6 cDNA was provided by Dr. Kremer, GSF, Munich, Germany. Monoclonal antibody against macrophage iNOS was obtained from Transduction Laboratories (Lexington, KY); antisera against the macrophage antigen F4/80 was from Serotec LTD (Wiesbaden, Germany); cell culture media (RPMI 1640, DMEM), fetal calf serum (FCS), penicillin/streptomycin, and TRIzol™ were from Life Technologies, Inc. (Eggenstein, Germany); cGMP radio-immunoassay kit (cGMP 153- assay system) and [3H]-arginine (60 Ci/ mmol), ECL detection system, and random primer labeling system (Rediprime™) were from Amersham (Braunschweig, Germany); Dowex 50 WX8 (Na+ form) was obtained from Serva (Heidelberg, Germany); [α-32P]UTP (800 Ci/mmol), [γ-32P]ATP, and [α-32P]dCTP (both 3000 Ci/ mmol) were from Boehringer Ingelheim Bioproducts (Heidelberg, Germany); IRF-1 binding oligonucleotide was from Santa Cruz Biotechnology; dexamethasone solution was ordered from Dr. Herschman, UCLA; TNF-α (10 ng/ml) was provided by Dr. Kleinert, University of Mainz, Germany; COX-2 cDNA was a gift from Dr. Kremer, GSF, Munich, Germany. T3/77 RNA polymerase transcription system was obtained from Stratagene (Heidelberg, Germany); dexamethasone solution was ordered from Boehringer Ingelheim Bioproducts (Heidelberg, Germany); IRF-1 binding oligonucleotide was from Santa Cruz Biotechnology (Heidelberg, Germany); Bradford protein assay was from Bio-Rad (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 (9) and were 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. Cells were from Dr. Dr. Kremer, GSF, Munich, Germany. All other materials were purchased from either Sigma (Deisenhofen, Germany) or ICN Biomedicals (Eschwege, Germany). All other materials were purchased from either Sigma (Deisenhofen, Germany) or ICN Biomedicals (Eschwege, Germany).

**Measurement of cGMP**—Confluent BMM or ABAE (24-well tissue plates) were washed three times and pretreated with 3-isobutyl-1-methylxanthine (0.5 mM) in serum-free RPMI 1640 for 10 min at 37 °C. Various stimulants were added for 30 min. Thereafter, medium was aspirated and cGMP was extracted immediately by the addition of HCl (0.1 N). After 10 min of incubation on ice the cell extracts were transferred to fresh tubes, lyophilized, and assayed for cGMP content by radioimmunoassay using a commercially available kit.

**Nitré Accumulation**—BMM (24-well plates, 200 μl) were treated with lipopolysaccharide (LPS, E. coli, serotype 055:B5, 1 μg/ml) in the presence or absence of various concentrations of ANP 99-126 and/or HS-142-1. After 20 h the stable metabolite of NO, nitrite, was measured in the presence or absence of Griess reaction buffer (22). 100 μl of cell extract was transferred to fresh tubes, lyophilized, and assayed for NO2− content by spectrophotometric measurement at 550 nm (reference wavelength 620 nm).

**iNOS Enzyme Activity**—BMM (24-well plates) were either untreated, stimulated with LPS (1 μg/ml) only, or co-incubated with ANP 10 nM−1 (μM) for 12 h, washed three times with cold PBS, frozen immediately, and stored at −70 °C. iNOS activity was determined by measuring the conversion of [14C]-arginine to [14C]-citrulline according to Ref. 23. Briefly, cells were homogenized in 50 μl Tris, pH 7.6, containing EDTA (0.1 mM), EGTA (0.1 mM), and phenylmethylsulfonyl fluoride (PMSF, 1 mM). After centrifugation at 10 000 g for 10 min at 4 °C to remove cellular debris, sonication lysates were either boiled for 5 min (fully denaturing conditions) or not boiled (partially denaturing conditions) to discriminate between iNOS dimer and monomer (24). Samples (60 μl of protein) were loaded on an SDS-polyacrylamide gel (7.5%) and electroblotted, and iNOS protein was detected using an anti-iNOS monoclonal antibody and the ECL detection system. Signal intensities were evaluated by densitometric analysis (Herolab, E.A.S.Y. plus system, Wiesloch, Germany).

**Detection of iNOS mRNA**—BMM were stimulated with or without LPS (1 μg/ml) in the presence or absence of ANP (1 μM) or dexamethasone (10 μM) for 6 h (24-well plates). RNA was prepared using TRIzol™ reagent and pooled from 6 wells. Northern blot was performed in principle as described previously (6, 9). Membranes were hybridized to a 32P-labeled murine macrophage iNOS cRNA probe (2 × 106 cpm/ml). iNOS cDNA (558 base pairs) was subcloned in a pBluescript SK+ vector, linearized (HindIII), and labeled with [α-32P]UTP (50 μCi) using a T3 RNA polymerase transcription system. Signal intensity was evaluated by densitometric analysis. Control for comparable amounts of intact mRNA loaded on the gel, membranes were rehybridized with a 32P-labeled β-actin probe (2 × 106 cpm/ml) as described (6).

**Analysis of mRNA Stability**—BMM in 24-well plates were treated with LPS (1 μg/ml) or a combination of LPS (1 μg/ml) plus ANP 99-126 (1 μM) for 5 h before addition of actinomycin D (5 μg/ml). Total RNA was prepared as described previously and further processed for Northern blot hybridization. iNOS cDNA was labeled with [α-32P]UTP (50 μCi) using a T3 RNA polymerase transcription system. Signal intensity was evaluated by densitometric analysis. Control for comparable amounts of intact mRNA loaded on the gel, membranes were rehybridized with a 32P-labeled β-actin probe (2 × 106 cpm/ml) as described (6).

**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 99-126 (1 μM-10 μM) for 2 h. Dibutyryl-cGMP (1 mM) or pyrrolidinedithiocarbamate (PDTC, 50 μM) was added 1 h prior to LPS stimulation. Nuclear extracts were prepared as described (29). Briefly, cells were incubated with PBS, resuspended in 400 μl of hypertonic buffer (110 mM HEPES, pH 7.9, 10 mM KC1, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF), and were allowed to swell for ice on 15 min. The supernatant was removed, and the nuclear pellet was extracted with 50 μl of hypertonic buffer (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM EDTA, 1
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The homogenate of LPS (1 mg/ml) was used to determine the protein concentration, which was determined at 12,000 x g, and the supernatant was centrifuged at 70 °C. The protein concentration was determined by the Lowry method (30).

Electrophoretic Mobility Shift Assay (EMSA)—Two 22-mer double-stranded oligonucleotide probes containing a consensus binding sequence for either NF-κB (5'-ACTTGAGGGGACCTTCCGAGGC-3') or IFN-1 (5'-GGAGGCGCAAATAATTGACT-3') were 5'-end-labeled with [γ-32P]-ATP (10 µCi) using T4 polynucleotide kinase. 10 µg 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 x 10^4 cpm radiolabeled oligonucleotide probe, 2 µg of poly(dI-dC), 4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 50 mM NaCl, and 0.5 µM DTT. Nucleotides and nucleotide complexes were resolved by electrophoresis (5%) non-denaturing 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 NF-κB and AP-2 (5'-GATCGAAGCTGACCGCCGGCGCCGT-3') binding sequences, respectively.

RESULTS

Demonstration of Guanylate Cyclase-coupled NPR-A in BMM, Its Implication in ANP-induced NO Inhibition—Previous data have been confirmed by a representative experiment shown in Fig. 1A demonstrated that ANP dose-dependently inhibits nitrite accumulation of LPS-activated BMM. The ANP-induced NO inhibition seems to be mediated by the guanylate cyclase-coupled NPR-A receptor, since HS-142-1, an NPR-A antagonist (31), abrogated the ANP effect. In addition, expression of the mRNA coding for both of the ANP receptors (NPR-A and NPR-C) has been shown before in BMM (10). The aim here was to clarify the actual presence of functional NPR-A receptor in BMM. BMM were treated with ANP, and cGMP production was determined. Intracellular cGMP levels were significantly elevated in cells exposed to ANP (10 µM-10 mM) for 30 min compared with untreated cells (Fig. 1B). HS-142-1 (100 µM) was able to antagonize this increase. To exclude that elevated cGMP levels were due to enzymatic activity of soluble guanylate cyclase (sGC) we incubated the cells with sodium nitroprusside (SNP), which is able to release NO, a known activator of sGC (14, 15). SNP (100 µM) did not change intracellular cGMP levels in BMM (Fig. 1C). In contrast, cGMP production of ABAE, known to express sGC (32), was dose-dependently elevated by the addition of SNP (10 and 100 µM) under identical conditions. Thus, BMM release NO when activated but do not possess sGC as an important target system for NO action.

iNOS Activity in Cell Homogenates—Suppression of nitrite accumulation in BMM by ANP has to be discussed as the sum of potentially cumulative actions of ANP on the iNOS system and, for instance, does not allow us to assess effects of ANP on specific enzyme activity. Therefore, ANP (1 µM) was added to the homogenate of LPS (1 µg/ml)-stimulated cells, and iNOS enzyme activity was measured as conversion rate of [3H]-arginine to [3H]-citrulline (Fig. 2A). Incubation of cell homogenate with ANP did not result in a change of iNOS activity. The cGMP analog dibutyl-cGMP (1 mM) had no effect either. The known specific inhibitor of NOS enzyme activity N6-monomethyl-L-arginine (3 mM) served as control and was able to inhibit specific iNOS enzyme activity up to 90%. By having excluded a direct effect of ANP on the enzyme activity, we next wanted to examine whether reduction of NO synthesis by ANP is due to factors such as decreased uptake of L-arginine or availability of other necessary substrates and cofactors for iNOS. By using an indirect approach, measurement of enzyme activity was performed with cell homogenates of ANP-pre-treated cells in the presence of optimal concentrations of substrates and cofactors (i.e. NADPH, L-arginine, BH4, FAD, and FMN). ANP pretreatment of cells dose-dependently (10 µM to 10 nM) reduced iNOS activity up to 70% (1 µM) compared with the activity of only LPS-stimulated cells (Fig. 2B). Thus, ANP induces inhibition of NO synthesis most likely not by causing an intracellular shortage of iNOS substrates. [3H]-Citrulline formation in the homogenate of ANP-exposed cells co-treated with the ANP-receptor antagonist HS-142-1 (10 µM) was almost restored completely to levels of LPS treatment only (Fig. 2B).

ANP Treatment Reduces iNOS Protein Expression—The reduced activity of iNOS after ANP treatment reflected a de-
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The next question was to determine whether ANP influenced the ratio of catalytically active iNOS dimer versus inactive monomer. Western blots were performed after partially denaturing conditions that allow detection of iNOS dimer (260 kDa) (24). Signal intensity of both dimer and monomer diminished in ANP-treated cells, but the proportion of iNOS dimer (260 kDa) (24) appeared when fully denaturing conditions were used for sample preparation. The expression of iNOS protein was significantly reduced by ANP (1 μM), whereas co-incubation with the NPR-A receptor antagonist HS-142-1 (100 μM) caused a marked reduction of LPS-induced iNOS mRNA accumulation.

**ANP Accelerates iNOS mRNA Decay**—The ANP-induced reduction of iNOS mRNA could be due to changes in either transcription or in mRNA stability. The effect of ANP on stability of iNOS mRNA was assessed by experiments employing the transcription inhibitor actinomycin D. BMM were stimulated with LPS (1 μg/ml) in the presence or absence of ANP (1 μM) for 5 h. Thereafter, actinomycin D was added (5 μg/ml), and total RNA was extracted at the indicated times and examined by Northern blot analysis. In order to evaluate the specificity of the ANP effect for iNOS mRNA blots were hybridized with a probe for COX-2, TNF-α, and IL-6 mRNA, respectively (Fig. 5A). These mRNAs were chosen for their similarity to the iNOS mRNA, i.e. they are inducible and contain AUUUA sequences in their 3′-untranslated region, known to be important for mRNA degradation (34, 35). To assess for equal loading of RNA and to normalize the amount of RNA in each sample, blots were rehybridized with a probe for β-actin (Fig. 5A). Autoradiographs were subjected to densitometry and evaluated as described under “Experimental Procedures.” The signal density ratio of iNOS/β-actin mRNA at each time expressed as percentage of that of the control (0 h) was plotted against time (Fig. 5B). It showed that the half-life of iNOS mRNA in ANP-treated macrophages was considerably decreased (1.9 versus 1.1 h un-

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**Fig. 2. Effects on iNOS activity.** A, l-citrulline formation as parameter for enzyme activity in homogenate of BMM stimulated with LPS (1 μg/ml, 12 h) was set as 100% (open bar). ANP (1 μM), dibutyryl-cGMP (Dibut., 1 mM), and N6-monomethyl-l-arginine (3 mM), respectively, were added to the reaction mixture composed as described under “Experimental Procedures,” and conversion of l-[3H]arginine to l-[3H]citrulline was determined. ANP (1 μM) treatment (Welch test). B, iNOS activity in homogenate of ANP-pretreated cells. BMM were either treated with LPS (1 μg/ml) alone (100%) or co-incubated with ANP (1 μM-10 nM) in the presence or absence of HS-142-1 (10 μg/ml) for 12 h. Cell homogenates were prepared and l-[3H]citrulline formation was determined as described under “Experimental Procedures.” Bars in both panels represent the mean ± S.E. of three independent experiments, each performed in triplicate. Statistical difference were as follows: **, p < 0.0001; *, p < 0.01 referring to the value after LPS treatment alone; +, p < 0.01 refers to LPS + ANP (1 μM) treatment (Welch test).

**Fig. 3. Western blot analysis of iNOS in LPS-activated BMM.** Detection of iNOS was performed with a specific monoclonal antibody in lysates of BMM either untreated (Co), stimulated with LPS (1 μg/ml), or co-treated with LPS (1 μg/ml) and ANP (1 μM) with or without HS-142-1 (100 μg/ml) for 12 h. The lysates were loaded on the gel (7.5% SDS-polyacrylamide gel electrophoresis) after boiling for 5 min (shown in A) or without boiling (demonstrated in B). Positions of iNOS monomers (150-kDa) and iNOS dimers (260-kDa) are indicated. Representative blots out of three experiments with similar results are shown.
treated versus ANP-treated cells). Evaluation of the signal intensities of the other mRNAs based on the autoradiographs shown in Fig. 5A, revealed no reduction of mRNA stability by ANP for TNF-α and IL-6 mRNA, respectively (data not shown). COX-2 mRNA decay, however, was increased by ANP similar to iNOS mRNA (Fig. 5C).

**ANP Inhibits NF-κB Binding Activity—**As reduction of iNOS mRNA stability may not be the only mechanism by which ANP reduces NO synthesis, we determined its effect on binding activity of NF-κB, the transcription factor known to be essential for iNOS induction in mouse macrophages (36). NF-κB binding activity of nuclear extracts was assessed by EMSA after stimulation of cells with LPS (1 μg/ml) for 2 h (Fig. 6). Formation of the specific DNA probe-NF-κB complex was dose-dependently reduced when nuclear extracts of cells co-incubated with ANP (10 nM–1 μM) were employed. The cGMP analog dibutyryl-cGMP (1 mM) could mimic the effect. The known inhibitor of NF-κB binding activity, PDTC (50 μM), was used as a control (36). Binding specificity was determined by addition of a 100-fold excess of unlabeled NF-κB or AP-2 binding sequence. For further control, binding activity of IRF-1, a transcription factor involved in INF-γ-induced iNOS expression (37), was tested. No IRF-1-DNA complex formation could be demonstrated in LPS-treated BMM (data not shown).

**Autocrine Mechanism of ANP-induced NO Inhibition—**By learning that ANP is able to interfere with iNOS expression, the question of its functional significance arises. It has previously been shown that ANP synthesis and secretion is markedly increased in LPS-activated BMM (9, 38), and thus, our working hypothesis was that ANP inhibits NO production via an autocrine mechanism.

We investigated whether blocking the NPR-A activation through endogenous ANP with the specific antagonist HS-142-1 would have an effect on LPS-induced NO secretion measured by nitrite accumulation. As shown in Fig. 7 treatment of BMM with HS-142-1 alone did not induce NO production as compared with untreated cells (Co). LPS (1 μg/ml) induced a marked NO production (100%) that was elevated dose-dependently by co-treating the cells with HS-142-1 (100 and 10 μg/ml).

**DISCUSSION**

This paper focuses on the regulation of iNOS expression by ANP in LPS-activated macrophages. ANP inhibits NO synthesis via the guanylate cyclase-coupled NPR-A receptor. Evidence is provided that inhibition by ANP is regulated at the transcriptional and post-transcriptional level as follows. 1) ANP did not affect the catalytic activity of iNOS. 2) Stability of iNOS mRNA was decreased in ANP-treated cells. 3) ANP inhibited binding activity of NF-κB, the predominant transcription factor for iNOS induction in LPS-activated murine macrophages (36).
Furthermore, cGMP is suggested to be the second messenger of the ANP effect based on the following data. 1) Exposure of BMM to ANP results in increased cGMP production. The increase of cGMP originated from activation of the particulate guanylate cyclase (NPR-A) rather than the soluble guanylate cyclase, which is not present in BMM (Ref. 39 and present data). 2) A specific NPR-A receptor antagonist (HS-142-1) (31) was shown to abrogate, whereas stable cGMP analogs could mimic the effects of ANP on various levels of iNOS regulation. Some information about the influence of cyclic nucleotides on iNOS expression are already available in the literature. In fact, natriuretic peptides via increased cGMP have been shown to augment IL-1-induced iNOS expression in vascular smooth muscle cells (40), and an interaction of cyclic GMP elevating agents with the cyclic AMP pathway has been suggested in this context (41). On the other hand, 8-BrcGMP was recently shown to inhibit iNOS expression and NO production in LPS-activated J774 macrophages (42). These data together with our results argue that the effect of cGMP on iNOS seems to be cell- and probably stimulus-dependent. A similar phenomenon has been reported for transforming growth factor-β, i.e. it inhibits iNOS synthesis in macrophages but induces iNOS in 3T3 fibroblasts (18, 43).

We found that ANP does not directly interact with the iNOS protein to reduce NO synthesis. This is in accordance with our previous observation that exogenous ANP is basically not taken up by BMM (9). A receptor-mediated effect on the catalytic site of iNOS could be excluded as well, since the cGMP analog did not affect the enzyme activity. However, iNOS activity was significantly reduced in the homogenate of ANP-exposed cells. Several mechanisms could account for this observation: ANP may stimulate substrate-degrading pathways such as arginase. This is unlikely since arginase activity has been inhibited in our experiments by addition of L-Valine (44). Furthermore, ANP might activate the induction of yet unidentified iNOS inhibitors. The Western blot analysis showing a strikingly decreased amount of iNOS protein, however, draws attention to sites of regulation other than enzyme activity which might be affected by ANP.

In this regard a major post-translational mechanism of iNOS regulation has been demonstrated to be intracellular dimerization of the iNOS molecule (24). iNOS is catalytically active only in the dimeric form. Dimer formation seems to be dependent on the availability of tetrahydrobiopterin, heme, and L-arginine (24). Interestingly, NO regulates its own synthesis by limiting intracellular assembly of iNOS by preventing heme insertion and decreasing heme availability (45). We addressed this rarely investigated regulatory mechanism of iNOS and could not provide evidence that ANP alters the extent of iNOS dimerization.

Effects of ANP on other regulatory mechanisms at the protein level such as translational activity or protein stability certainly cannot be ruled out since the corresponding experiments have not been performed in this study. However, based on the data obtained by Northern blot analysis, the decreased amount of iNOS protein is most likely a consequence of the significantly lower concentration of iNOS mRNA observed in ANP-exposed cells.

Levels of mRNA are controlled via transcriptional activity and via mRNA stability. mRNA degradation seems to be an important regulatory mechanism for iNOS expression (12, 17, 18) as the iNOS gene contains AUUUA sequences within its 3′-untranslated region known to be responsible for the instability of mRNA (for review see Ref. 28). This sequence represents a binding motif for short-lived RNase that attaches to this site and is responsible for the extreme lability of the corresponding mRNAs (46). Importantly, ANP reduces stability of iNOS mRNA in BMM. Half-life of iNOS mRNA in ANP-exposed cells was reduced around 50% as compared with only LPS-activated cells. In order to elucidate whether ANP specif-
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influence on the cardiovascular hormone ANP. By inhibition of NF-κB activation via cGMP and destabilization of iNOS mRNA, ANP specifically interacts with NO production of LPS-activated macrophages.

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**Mechanism of ANP-induced NO Inhibition in Macrophages**

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