

# Atrial Natriuretic Peptide, a Regulator of Nuclear Factor- $\kappa$ B Activation *in Vivo*

Kathrin Ladetzki-Baehs,\* Melanie Keller,\* Alexandra K. Kiemer, Elke Koch, Stefan Zahler, Albrecht Wendel, and Angelika M. Vollmar

Department of Pharmacy, Center of Drug Research (K.L.-B., M.K., E.K., S.Z., A.M.V.), University of Munich, 81377 Munich, Germany; Department of Pharmacy (A.K.K.), Saarland University, 66041 Saarbrücken, Germany; and Biochemical Pharmacology (A.W.), University of Konstanz, 78457 Konstanz, Germany

Natriuretic peptides (NPs) comprise a family of vasoactive hormones that play important roles in the regulation of cardiovascular and renal homeostasis. Along this line, atrial NP (ANP) (international non-proprietary name: carperitide, HANP) is an approved drug for the treatment of acute heart failure. In recent years, evidence has been given that the NP system possesses a far broader biological spectrum than the regulation of blood pressure and volume homeostasis. In fact, a substantial amount of *in vitro* work indicates that ANP affects important inflammatory processes and signaling pathways. Quite surprisingly, however, no information exists on the *in vivo* antiinflammatory potential and signaling of ANP.

We show here that pretreatment of lipopolysaccharide (*Salmonella abortus equi*, 2.5 mg/kg)-challenged mice with ANP (5  $\mu$ g/kg iv, 15 min) rapidly inhibits nuclear factor- $\kappa$ B activation via inhibition of phosphorylation and degradation of the I $\kappa$ B- $\alpha$  protein. ANP also reduces Akt activation upon lipopolysaccharide injection. In ANP-pretreated mice, the increase of TNF- $\alpha$  serum concentration is markedly prevented; most importantly, the survival of these animals improved. These findings demonstrate both *in vitro* and *in vivo* an antiinflammatory profile of ANP that deserves to be further investigated in a therapeutic perspective. (*Endocrinology* 148: 332–336, 2007)

WHEN LIPOPOLYSACCHARIDE (LPS), a major constituent of the outer membrane of Gram-negative bacteria, crosses the natural barriers of the body and reaches the systemic circulation, it might give rise to a systemic inflammatory response (1). The induction of proinflammatory gene expression by LPS is an important aspect in the immunopathological features of LPS-induced shock (2). The transcription factor nuclear factor (NF)- $\kappa$ B is thought to be one of the central regulators of this process (3). NF- $\kappa$ B is composed of homo- and heterodimers of the Rel family proteins, such as p65 and p50. NF- $\kappa$ B is held in an inactive form in the cytoplasm by association with members of the I $\kappa$ B family. Phosphorylation and degradation of I $\kappa$ B in response to LPS leads to NF- $\kappa$ B translocation into the nucleus (3, 4). NF- $\kappa$ B activation plays an indispensable role in inducing the rapid production of TNF- $\alpha$  (3). This cytokine in turn activates a second level of inflammatory cascades, leading to vascular instability and microvascular occlusion that might contribute to septic multiple organ failure (3). Therefore, NF- $\kappa$ B and TNF- $\alpha$  have widely been proposed as therapeutic targets in critical care medicine (3, 5–8), and safe, efficient, and specific inhibitory compounds are urgently searched for. The cardiovascular peptide, atrial natriuretic peptide (ANP), represents a promising candidate because it has been shown *in*

*vitro* that the peptide inhibits both activation of NF- $\kappa$ B and TNF- $\alpha$  production in mouse macrophages (9, 10).

ANP belongs to a family of structurally related 22–53-amino acid peptides, which display broad and disparate effects in the cardiovascular system (11). Importantly, ANP (international non-proprietary name: carperitide, HANP) is in fact approved as a drug in Japan for the treatment of acute heart failure (12). Notably, we and others demonstrated that natriuretic peptide (NP) biology is not restricted to cardiovascular functions but is linked to the immune system. NPs, especially ANP and their receptors, are expressed and regulated in immune cells (13). Moreover, both ANP and ANP receptors are regulated by inflammatory stimuli, suggesting NPs as regulatory proteins in inflammatory processes (13, 14). ANP is not only regulated by proinflammatory factors but also exerts antiinflammatory action on isolated macrophages as well as on endothelial cells interfering with the MAPK network as well as with proinflammatory transcription factors, such as NF- $\kappa$ B and activating protein-1 (9, 15, 16). However, until now, the *in vivo* relevance of this mechanistic work on ANP has not been examined. Here, we report that administration of ANP potently abrogates LPS-induced NF- $\kappa$ B activation and prevents increased TNF- $\alpha$  serum concentration and thereby improves survival of mice exposed to bacterial LPSs.

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\* K.L.-B. and M.K. contributed equally to this study.

Abbreviations: ANP, Atrial natriuretic peptide; BW, body weight; HPRT, hypoxanthine phosphoribosyltransferase; LPS, lipopolysaccharide; NF, nuclear factor; NP, natriuretic peptide; PMSF, phenylmethylsulfonyl fluoride.

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## Materials and Methods

### Animal model

Male pyrogen-free BALB/c mice (20–30 g) were injected with LPS [2.5 mg/kg body weight (BW) *Salmonella abortus equi* S.; Bioclot, Aidenbach, Germany]. Mice were treated with either ANP (5  $\mu$ g/kg BW iv; Bachem, Heidelberg, Germany). ANP dosage is based on preliminary dose curve experiments measuring effects on TNF- $\alpha$  serum concentration) or NaCl

solution (0.9%) 15 min before LPS challenge. Survival of mice was monitored for at least 72 h. In another set of experiments, organ and blood samples were obtained after lethal iv anesthesia (pentobarbital, Sanofi-Ceva, Düsseldorf, Germany; 0.8 mg/kg BW heparin, Braun, Melsungen, Germany) at different time points (15, 30, 90 min) and immediately snap-frozen in liquid nitrogen. Blood was withdrawn by cardiac puncture. Animal experiments have been performed with the permission of the government authorities and in accordance with the German legislation on Laboratory Animal Experiments. All studies followed the directives of the University of Konstanz Ethical Committee.

### TNF- $\alpha$ measurement

Serum and tissue TNF- $\alpha$  levels were determined by ELISA using an OptEIA Mouse-Specific TNF- $\alpha$  ELISA Set (mono/mono; BD Biosciences, Heidelberg, Germany).

For TNF- $\alpha$  mRNA expression, total RNA contents were extracted using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instruction manual. Real-time RT-PCR was performed with the Dynamo Probe 2-Step qRT-PCR Kit (Finnzymes; New England Biolabs, Espoo, Finland). The following primers and probes (Biomers, Ulm, Germany) have been used: TNF- $\alpha$  forward (400 nm), 5'-TGG CCT CCC TCT CAT CAG TTC-3'; TNF- $\alpha$  reverse (400 nm), 5'-TTG GTG GTT TGC TAC GAC GTG-3' (bp 1–1561; GenBank accession no. NM 013693); TNF- $\alpha$  TaqMan probe (100 nm), 5'-FAM-TGG CCC AGA CCC TCA CAC TCA GAT CAT C-BHQ-3'; hypoxanthine phosphoribosyltransferase (HPRT)1 forward, 5'-GTT AAG CAG TAC AGC CCC AAA ATG-3'; HPRT1 reverse, 5'-AAA TCC AAC AAA GTC TGG CCT GTA-3' (bp 1–1261; GenBank accession no. NM 013556); and HPRT1 TaqMan probe, 5'-HEX-AGC TTG CTG GTG AAA AGG ACC TCT CGA AGT-BHQ-3'. HPRT was used as an internal housekeeping gene. Calculation of the mRNA content was performed by a mathematical model developed by Pfaffl (17).

### DNA binding of NF- $\kappa$ B: EMSA analysis

Tissue was homogenized (Potter S, B. Braun Biotech) in buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonylfluoride (PMSF)], centrifuged, and further incubated (4 C, 10 min) in buffer A containing 10% NP-40. Probes were centrifuged, the supernatant was discarded, and the remaining pellet was suspended in buffer B [20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 1 mM dithiothreitol, 0.1 mM PMSF]. Samples were kept at 4 C for 30 min while continuously shaking. After centrifugation, supernatants were frozen at  $-80$  C. EMSA was performed as described previously (18). The consensus binding sequence used for NF- $\kappa$ B was 5'-AGT TGA GGG GAC TTT CCC AGG C-3' (Promega, Mannheim, Germany). Specificity of the protein-DNA complex was confirmed by adding a 100-fold excess of unlabeled NF- $\kappa$ B or activating protein-1 (5'-CGC TTG ATG AGT CAG CCG GAA-3') binding sequences.

### Western blot

Liver tissue (20–60 mg) was homogenized (Potter S, B. Braun Biotech) in 200–600  $\mu$ l lysis buffer [50 mM HEPES, 50 mM NaCl, 5 mM EDTA, 10 mM NaPyroP, 50 mM NaF, 1 mM PMSF, 1 mM Na-vanadate (pH 7.0), 1% Triton X-100, 1 $\times$  Complete]. After centrifugation, samples were used for protein quantification by use of the Pierce assay (BC assay reagents; Interdim, Montluçon, France). Proteins were separated by electrophoresis and subsequently transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). Proteins were identified by incubating the membrane with primary antibody [ $\text{I}\kappa\text{B-}\alpha$ , Santa Cruz, Heidelberg, Germany;  $\text{I}\kappa\text{B-}\alpha$  phospho (Ser32/Ser36), Akt phospho (Ser473), Akt, Cell Signaling, Frankfurt/Main, Germany; at 4 C overnight], followed by incubation with a horseradish peroxidase-conjugated secondary antibody (goat antirabbit; Dianova, Hamburg, Germany). The reactive bands were visualized employing a chemiluminescent detection kit (ECL Plus; Amersham Pharmacia, Uppsala, Sweden).

### Statistical analysis

Animal experiments were performed using the number of animals as shown in the respective figure legend. Results are shown as mean  $\pm$  SEM. Statistical analysis as indicated was performed using GraphPad Prism 3.03 software (GraphPad Software Inc., San Diego, CA). Significance was calculated at the 95% confidence interval in comparison with its corresponding control experiment, unless indicated otherwise.

## Results

### ANP improves survival of mice challenged with LPS

Administration of LPS (2.5 mg/kg BW) alone caused mortality of all mice within 24 h. In contrast, 75% of the animals receiving ANP (5  $\mu$ g/kg BW) 15 min before lethal LPS injection survived (Fig. 1A).

### ANP prevents LPS-induced increase of serum TNF- $\alpha$ level

TNF- $\alpha$  concentration in blood is an important marker in LPS-induced shock. Ninety minutes after LPS injection, LPS-induced TNF- $\alpha$  serum levels were reduced by 88% in ANP-pretreated mice compared with control mice (Fig. 1B). Administration of ANP alone did not affect TNF- $\alpha$  concentration (data not shown).

### ANP decreases TNF- $\alpha$ tissue expression

Examining various tissues for effects of ANP on TNF- $\alpha$  synthesis revealed that spleen and even more pronounced

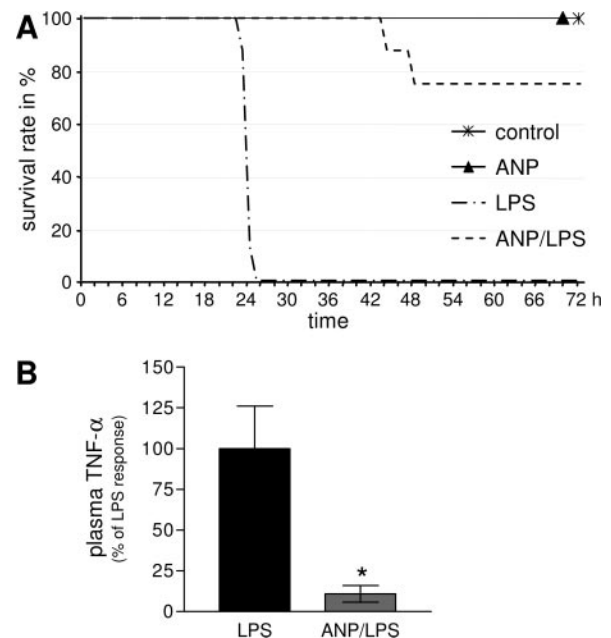


FIG. 1. A, ANP pretreatment reduces mortality in LPS-challenged mice. Animals were pretreated with ANP (5  $\mu$ g/kg BW) for 15 min before LPS (2.5 mg/kg BW) injection. NaCl as well as solely ANP administration were used for control. The animal's state of health was continuously observed for 72 h. Eight animals were analyzed for each treatment group. Graph displays survival rate in percentage of control mice (\*), ANP-treated mice (▲), LPS-treated mice (- · -), and ANP-pretreated LPS-treated mice (- - -). B, ANP prevents increase of serum TNF- $\alpha$  levels in LPS-treated mice. Animals were treated as mentioned under A. After 90 min of LPS injection, blood was withdrawn from the heart and used for TNF- $\alpha$  (concentration) determination by ELISA. Statistical analysis was performed by data evaluation using the unpaired Student's *t* test, implicating analysis of means  $\pm$  SEM. \*, *P* < 0.05.

liver show a rapid (after 30 min) decrease of LPS-induced TNF- $\alpha$  gene expression after ANP pretreatment (Fig. 2, C and D). ANP injection as such did not alter basal TNF- $\alpha$  expression (data not shown). TNF- $\alpha$  mRNA expression in heart and lung was not affected by ANP (Fig. 2, A and B). TNF- $\alpha$  protein concentration was reduced in lung (by  $32 \pm 9\%$ ), liver (by  $34 \pm 15\%$ ), and spleen ( $70 \pm 11\%$ ) in ANP-pretreated animals compared with LPS-challenged animals (90 min).

#### Impact of ANP on the transcription factor NF- $\kappa$ B

The transcription factor NF- $\kappa$ B is one of the most important players within the LPS-induced signaling cascade, finally leading to TNF- $\alpha$  expression. As shown in Fig. 3, endotoxemia immediately led to highly increased NF- $\kappa$ B binding activity, detectable as soon as 15 min after LPS injection in whole-liver homogenates. NF- $\kappa$ B binding activity has not been detectable in NaCl (control)-treated mice, and ANP injection alone does not influence NF- $\kappa$ B activation. Notably, preconditioning with the cardiovascular hormone ANP provoked a strong decrease of NF- $\kappa$ B binding activity at both 15 and 30 min past LPS injection. This effect points to ANP being able to modulate one of the key parameters involved in early inflammatory signaling pathways.

#### Mechanisms leading to decreased NF- $\kappa$ B binding activity

Effects of ANP on phosphorylation and degradation of NF- $\kappa$ B inhibitory factor I $\kappa$ B- $\alpha$ , as well as on activation of the protein kinase Akt, in livers of LPS-treated mice were ex-

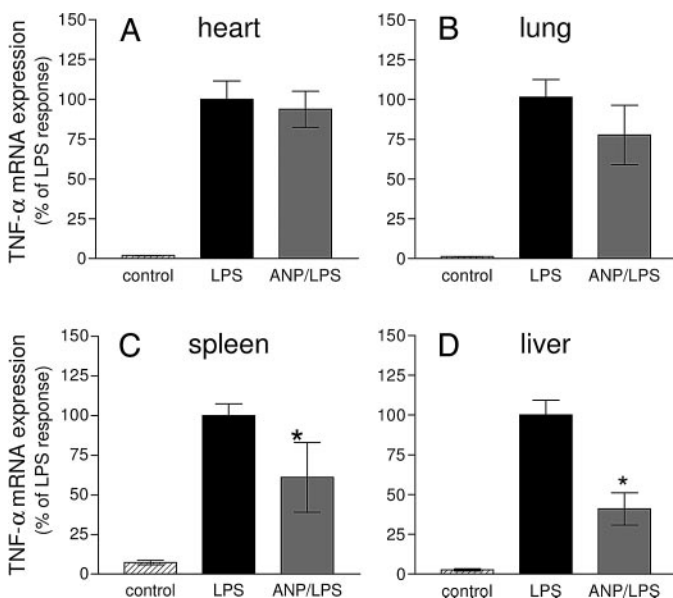


FIG. 2. ANP pretreatment modulates TNF- $\alpha$  mRNA expression in different organs derived from LPS-treated mice. Animals were pretreated with ANP ( $5 \mu\text{g}/\text{kg BW}$ ) as described in Fig. 1. NaCl administration was used for control experiments. Organ samples were obtained 30 min after LPS injection. Whole-organ homogenates were analyzed by real-time RT-PCR. Graphs are displaying TNF- $\alpha$  mRNA expression in heart (A), lung (B), spleen (C), and liver (D). Four animals were analyzed for each treatment group. Statistical analysis was made by evaluation of these data using the unpaired Student's *t* test, implicating analysis of means  $\pm$  SEM. \*,  $P < 0.05$ .

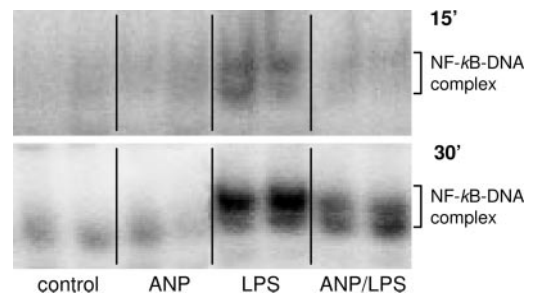


FIG. 3. ANP pretreatment reduces LPS-induced NF- $\kappa$ B binding activity in liver homogenates. Animals were pretreated with ANP ( $5 \mu\text{g}/\text{kg BW}$ ) 15 min before LPS injection. Mice exclusively receiving NaCl (control) or ANP were also examined. Organ samples were obtained 15 and 30 min after LPS injection. For investigations of NF- $\kappa$ B binding activity, whole-liver homogenates were analyzed by EMSA. EMSA assays were performed in duplicate.

amined. Both proteins are known to be involved in LPS-mediated NF- $\kappa$ B activation.

As shown in Fig. 4A, increased I $\kappa$ B- $\alpha$  phosphorylation and decreased levels of total I $\kappa$ B- $\alpha$  were clearly detected 30 min after LPS injection. Importantly, ANP pretreatment abrogated LPS-induced I $\kappa$ B- $\alpha$  phosphorylation as early as after 15 min compared with LPS-treated animals. An increase of total protein contents of I $\kappa$ B- $\alpha$  was not seen within the first 30 min in LPS- and ANP-treated livers. LPS administration led also to a significant increase in Akt phosphorylation in mouse liver after 30 min (Fig. 4B). ANP preconditioning clearly decreased this Akt phosphorylation. Interestingly, pretreatment of mice with wortmannin ( $16 \mu\text{g}/\text{kg}$ ), a common phosphatidylinositol 3-kinase inhibitor that leads to inhibition of Akt phosphorylation, also decreased TNF- $\alpha$  serum level in LPS-treated mice although not as potently as ANP pretreatment ( $42 \pm 14\%$  vs.  $88 \pm 4\%$ ).

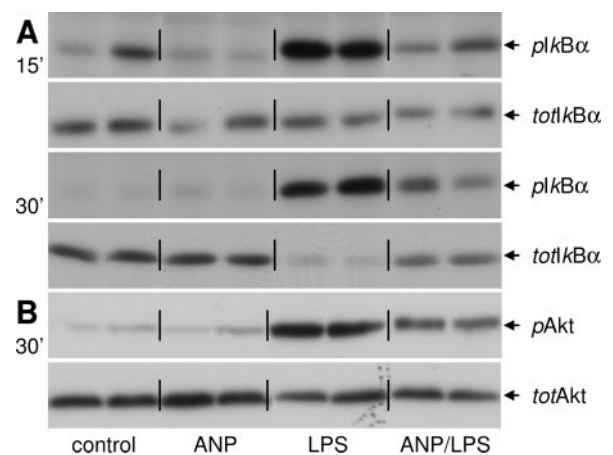


FIG. 4. ANP pretreatment reduces LPS-induced I $\kappa$ B- $\alpha$  phosphorylation, I $\kappa$ B- $\alpha$  degradation, and Akt phosphorylation in liver. Animals were pretreated with ANP ( $5 \mu\text{g}/\text{kg BW}$ ) 15 min before LPS injection ( $2.5 \text{ mg}/\text{kg BW}$ ). Mice receiving NaCl (control) or ANP were additionally examined. Liver was excised 15 and 30 min after LPS injection. I $\kappa$ B- $\alpha$  phosphorylation (pI $\kappa$ B $\alpha$ ) and I $\kappa$ B- $\alpha$  total protein contents (totI $\kappa$ B $\alpha$ ) (A) and Akt phosphorylation (pAkt) as well as Akt total protein contents (totAkt) (B) were determined by Western blot analysis. Representative blots of six independent experiments are shown.

## Discussion

This work profiles the cardiovascular peptide ANP as a potent regulator protein of NF- $\kappa$ B activation and, consecutively, TNF- $\alpha$  production *in vivo*. Pretreatment of mice with ANP resulted in improved survival of mice after LPS challenge. Although various *in vitro* data indicate an antiinflammatory profile of ANP, this is the first report of a potent *in vivo* antiinflammatory action of this peptide hormone, which is approved as a drug for acute heart failure.

We showed that ANP early and efficiently targets NF- $\kappa$ B activation, a central molecular event leading to the development of the systemic inflammatory response syndrome (3). NF- $\kappa$ B is activated by a variety of bacteria and bacterial components and is a common denominator of a plethora of shock-inducing agents. Studies using animal models of systemic inflammation have shown that NF- $\kappa$ B activity is markedly elevated in multiple organs (3, 19, 20). NF- $\kappa$ B activity is also strongly increased in patients with septic shock, and the degree of NF- $\kappa$ B activity correlates with the severity of the disease (19, 21). Greater NF- $\kappa$ B activity is associated with higher rates of mortality and worse clinical outcome. NF- $\kappa$ B activation mediates the transcriptional expression of a large number of proinflammatory proteins, such as TNF- $\alpha$ , which play important roles in the pathophysiology of systemic inflammation. Mice deficient in those NF- $\kappa$ B-dependent genes are resistant to the development of septic shock and sepsis-related death in endotoxin models (22, 23). Therefore, efficient inhibitors of NF- $\kappa$ B are searched for urgently. Along this line, I $\kappa$ B- $\alpha$  gene transfer strategies either overexpressing a dominant-negative or a wild-type isoform of I $\kappa$ B- $\alpha$  have turned out to be beneficial in *in vivo* animal studies (19). However, the application of systemic NF- $\kappa$ B inhibitors using genetic approaches faces problems such as feasibility, safety, and efficacy, which have yet to be overcome. Other known NF- $\kappa$ B inhibitors include proteasome inhibitors, glucocorticoids, nonsteroidal antiinflammatory drugs, antiinflammatory cytokines, and various natural products. However, they also face problems regarding their therapeutical application because efficacy, but also known (and unknown) side effects will limit their clinical use (3, 8). In this respect, ANP, being an endogenous peptide and approved as a drug for acute myocardial failure, has some advantages. The dose necessary to block NF- $\kappa$ B in our model is well within the limits of therapeutic doses given in case of acute heart failure (12, 24). However, because it is known that during septic shock severe hypotension is prevalent, the effect of ANP on blood pressure may be a limiting factor in the treatment. On the other hand, the usefulness of ANP may be analogous to its beneficial effect in the treatment of heart failure, which is a clinical situation with increased ANP.

Importantly, ANP pretreatment affects the LPS-induced NF- $\kappa$ B/TNF- $\alpha$  pathways at a rather upstream stage. These are important features regarding the therapeutic outcome. Transcriptional activation of NF- $\kappa$ B in LPS-challenged individuals results in a rapid release of TNF- $\alpha$  into the circulation and although reduction of TNF- $\alpha$  plasma levels leads to improved survival in a murine LPS-induced shock (25), anti-TNF- $\alpha$  strategies failed to prevent death in septic patients (26). This fact is probably related more to the difficulty of

designing clinical trials in these patients rather than to the scientific rationale. Anti-TNF- $\alpha$  therapy requires a prophylactic treatment to prevent the deleterious secondary inflammatory responses. Because we did not examine whether ANP will also lead to reduced TNF- $\alpha$  level when given simultaneously or even after LPS application to the peptide, we are not really able to comment on its role for the intervention in human septic shock patients. However, because of the distinct prevention of the increase of TNF- $\alpha$  serum levels by ANP, the peptide at least might exert considerable antiinflammatory action in humans.

Regarding the underlying signaling pathway, ANP is shown to abrogate LPS-induced phosphorylation of I $\kappa$ B- $\alpha$  and consecutively its degradation. ANP affects another target discussed in the pathology of sepsis, namely Akt. LPS exposure either of animals or patients leads to an increase of phosphorylated Akt that could even be predictive for the clinical outcome (21). ANP inhibits the LPS-induced activation of Akt. Akt is known to phosphorylate p65 and thereby to increase its transcriptional activity (27). ANP may inhibit NF- $\kappa$ B not only by inhibiting nuclear translocation of p65 but also via inhibition of its transcriptional activity. In fact, we observed reduced TNF- $\alpha$  levels by employing wortmannin, a PI3 kinase inhibitor that reduces Akt activity.

It is important to mention that inhibition of Akt is a two-edged sword effect and asks for caution regarding its general therapeutical use. Besides its proposed role in sepsis, Akt is discussed to promote tumor progression through increased cell survival. However, the protein Akt has recently been shown to be also closely implicated in the blockade of cancer cell motility and metastasis (28).

Our data do not only point to a potential therapeutic use of ANP as an antiinflammatory drug but also prompt the question about the role of the endogenous ANP system in sepsis. Until now, pro-ANP concentrations are used as reliable diagnostic and prognostic tools in the management of congestive heart failure and recently also of severe sepsis (29, 30) and discussed as a consequence of left ventricular systolic dysfunction. However, it has also been shown that immune cells such as macrophages but also endothelial cells secrete increased levels of NPs upon challenge with LPS (13, 14). Furthermore, ANP has been shown to inhibit inflammatory mediators such as inducible nitric oxide synthase via an autocrine mechanism (10). Thus, elevated ANP levels in sepsis might also function as an endogenous regulator of inflammation.

In summary, the data presented here show that ANP, a cardiovascular hormone used as a drug against heart failure, possesses potent antiinflammatory actions *in vivo*. Because ANP targets early NF- $\kappa$ B activation upon LPS challenge, we propose to consider this factor for intervention therapy strategies in systemic inflammation.

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Address all correspondence and requests for reprints to: Angelika M. Vollmar, Ph.D., Department of Pharmacy, Center of Drug Research, Butenandtstrasse 5-13, D-81377 Munich, Germany. E-mail: Angelika.Vollmar@cup.uni-muenchen.de.

The authors have nothing to disclose.

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