0013-7227/02/$15.00/0
Printed in U.S.A.

Endocrinology 143(3):846–852

Inhibition of Cyclooxygenase-2 by Natriuretic Peptides

ALEXANDRA K. KIEMER, MARTIN D. LEHNER, THOMAS HARTUNG, AND ANGELIKA M. VOLLMAR

Department of Pharmacy (A.K.K., A.M.V.), Center of Drug Research, University of Munich, 81377 Munich, Germany; and University of Konstanz (M.D.L., T.H.), Biochemical Pharmacology, 78457 Konstanz, Germany

The atrial natriuretic peptide (ANP) has been suggested to possess immunomodulatory potential because of its property to alter macrophage functions via its guanylate-cylcase-coupled A-receptor (NPR-A), such as inhibiting the expression of inducible nitric oxide synthase or TNF-α. The aim of this study was to investigate whether ANP influences COX-2. COX-2 expression in murine macrophages and in mice was induced by lipopolysaccharide. Release of PGE_2 and thromboxane B_2 was significantly reduced in the presence of ANP. C-type natriuretic peptide (CNP) also significantly reduced PGE_2-accumulation in macrophages. Northern and Western blots showed that ANP attenuates COX-2 mRNA and protein. Reduction of neither COX-2 nor of PGE_2 production was significantly abrogated by an NPR-A antagonist, suggesting a pathway independent of cGMP. Furthermore, dibutyryl-cGMP did not affect PGE_2-accumulation. cANF, the specific ligand for the natriuretic peptide (NP) clearance-receptor (NPR-C), significantly inhibited PGE_2-production. Because some biological activities of ANP have been reported to be mediated via an NPR-C-mediated inhibition of adenylate-cyclase, we determined cAMP levels. ANP, CNP, and cANF significantly attenuated intracellular cAMP. In summary, ANP was shown to attenuate PGE_2-production of lipopolysaccharide-activated macrophages predominantly via the NP clearance-receptor. ANP reduces COX-2-protein and -mRNA levels. The inhibition seems to be mediated via NPR-C and related to an attenuation of cAMP production. (*Endocrinology* 143: 846–852, 2002)

THE ATRIAL NATRIURETIC peptide (ANP) is a member of the natriuretic peptide (NP) family, a group of cardiovascular cyclic peptide hormones (1). The action of ANP in the cardiovascular system is well studied, and investigations concentrate mainly on the diuretic, natriuretic, and vasodilating properties of ANP (1–4). Most biological effects of ANP are mediated by the guanylate cyclase-coupled A-receptor (NPR-A) (5). However, an increasing number of reports describe that several effects of ANP are mediated via the NP so-called clearance receptor (NPR-C) (6). These effects seem to be related to a G protein-coupled inhibition of adenylate cyclase (3, 7).

The C-type NP (CNP), a further member of the NP family, is the specific ligand for the B-receptor (NPR-B), another guanylate-cyclase-coupled NP receptor (6). However, CNP, like ANP, also binds to the NPR-C (6). CNP is suggested to be the major NP in the brain (8), but its expression was also demonstrated in peripheral cells, such as endothelial cells (9, 10) and macrophages (11, 12). CNP possesses minor natriuretic and diuretic properties, compared with ANP (13). Therefore, the physiological role of CNP is suggested to be different from that of ANP.

It is increasingly recognized that the functions of the NPs are not restricted to the regulation of volume homeostasis. NPs and their receptors have been demonstrated to be expressed in diverse tissues besides the cardiovascular and renal system (14). We could previously demonstrate a link of ANP to the immune system, thereby providing novel aspects in the biological profile of NP (15). ANP and its receptors are expressed in thymus (16, 17), as well as in macrophages (12, 18, 19). ANP was also shown to exert various effects in the immune system. In this context, we demonstrated that ANP inhibits thymopoesis and thymocyte proliferation (17, 20). Moreover, ANP increases phagocytosis and respiratory burst of macrophages (21), and it reduces the production of TNF-α (22) in these cells. Importantly, ANP inhibits inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-activated macrophages (18, 19). ANP influences this proinflammatory enzyme, in an autocrine fashion, via its guanylate-cyclase coupled A-receptor (18, 19, 23). The potency of ANP to regulate macrophage functions leads us to suggest that ANP might play a role in inflammation.

Since some years, new antiinflammatory therapeutic strategies very strongly focus on the inducible form of the cyclooxygenase (COX), the COX-2 (24, 25). There are two isoforms of COX (COX-1 and COX-2) that catalyze the synthesis of PGs from arachidonic acid (26, 27). The constitutive isoform, COX-1, is expressed in most tissues and seems to be responsible for the production of PGs that mediate physiological functions, such as protection of the gastric mucosa and regulation of renal blood flow (27). COX-2, however, is not expressed in most normal tissues but is induced by endotoxin, cytokines, growth factors, oncogenes, and tumor promoters (28–31). It is increasingly accepted that COX-2 is an important therapeutic target for preventing or treating diseases like arthritis or cancer (24). COX-2 expression is increased in inflamed tissues, such as rheumatoid synovium (32), and selective inhibitors of COX-2 are successful therapeutics in the treatment of arthritis (25). Furthermore, COX-2 is up-regulated in transformed cells (33) and in tumors (24). Taken together, these data support the therapeutic strategy of controlling COX-2 to inhibit inflammation and carcinogenesis. To develop an effective approach for controlling

Abbreviations: ANP, Atrial natriuretic peptide; BMM, bone marrow macrophages; cANF, the specific ligand for the NP; COX, cyclooxygenase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NaCl/HSA, 0.9% NaCl with 0.1% wt/vol human serum albumin; NP, natriuretic peptide; NPR, NP receptor; PTX, pertussis toxin; TxB_2, thromboxane B_2.

COX-2, however, it is essential to understand the endogenous mechanisms that regulate the expression of COX-2.

The cardiovascular hormone ANP has been demonstrated to possess autocrine immunomodulatory potential *in vitro* (15, 19). Furthermore, the serum levels of the immunomodulatory hormone have been reported to be up-regulated in inflammatory conditions, such as septic shock (34) or arthritis (35). We hypothesized that ANP represents a physiological regulator of COX-2. To elucidate this hypothesis, we investigated the potential influence of ANP on COX-2 *in vitro* as well as *in vivo*. Our investigations should: 1) show whether ANP or CNP influence this central proinflammatory enzyme; 2) determine the type of receptor mediating a potential effect; and 3) give information on the underlying mechanism of COX-2 inhibition by ANP.

Materials and Methods

Experimental animals

All animal experimentation described in the manuscript was conducted in accord with accepted standards of humane animal care in accordance with the NIH guidelines and the legal requirements in Germany.

Materials

Rat ANP 99–126 (ANP) was purchased from Calbiochem/Novabiochem (Bad Soden, Germany); and CNP and cANF, from Saxon Biochemicals (Hannover, Germany). HS-142-1 was a gift from Dr. Matsuda, Pharmaceutical Research Institute, Kyowa Hakko Kogyo Co., LTD, Shizuoka, Japan. COX-2 cDNA was obtained from Dr. Herschman, University of California (Los Angeles, CA); antiserum against the macrophage antigen F4/80 was from Serotec (Wiesbaden, Germany); cell culture medium (RPMI 1640), FCS, penicillin/streptomycin, and TRIzol were from Life Technologies, Inc. (Karlsruhe, Germany). [α^{32}P]-UTP (800 Ci/mmol), the ECL system, and cAMP ELISA were from Amersham Pharmacia Biotech (Braunschweig, Germany); dexamethasone solution was ordered from Centravet (Bad Bentheim, Germany); T7 polynucleotide kinase and Complete were obtained from Boehringer Ingelheim GmbH Bioproducts (Heidelberg, Germany). Anti-COX-2 monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Bradford protein assay was from Bio-Rad Laboratories, Inc. (Munich, Germany), and the PGE_2 ELISA was from BioSource Technologies, Inc. (Nivelles, Belgium). All other materials were purchased from Sigma (Deisenhofen, Germany).

Cell culture

Mouse bone marrow macrophages (BMM) were prepared as described previously (36), seeded at a density of 2×10^5 cells/ml in 24-well tissue plates, and grown for 5 d (5% CO_2, 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 found more than 95% pure, as judged by fluorescence-activated cell sorter analysis (FACScan, Becton Dickinson and Co., Heidelberg, Germany), using an antiserum against the macrophage antigen F4/80.

PGE_2 ELISA

BMM (24-well plates, 200 μl) were untreated or treated with LPS (*Escherichia coli*, serotype 055:B5, 1 μg/ml). The effect of the following substances on PGE_2 production was determined: ANP (10^{-9}–10^{-6} M), CNP (10^{-8}–10^{-6} M), cANF (10^{-7}–10^{-6} M), dibutyryl-cGMP (10^{-3} M), and HS-142-1 (100 μg/ml). As previously shown, none of the substances in the used concentrations possesses cytotoxic activity on BMM. Substances were added to the cells simultaneously with LPS. After 20 h, PGE_2 accumulation was measured in the supernatant by a commercially available ELISA.

cAMP ELISA

BMM (24-well tissue plates) were washed three times and pretreated with 3-isobutyl-1-methylxanthine (100 μM) in serum-free cell culture medium RPMI 1640 for 10 min at 37 C. The cells were activated with LPS (1 μg/ml) and various stimuli (ANP, 10^{-8}–10^{-6} M; CNP, 10^{-6} M; cANF, 10^{-7}–10^{-6} M) were added for 30 min. Thereafter, medium was aspirated and assayed for cAMP content, by ELISA using a commercially available kit extracting cAMP by lysis buffer as described by the manufacturer's instruction.

In a second set of experiments, cells were pretreated overnight with pertussis toxin (PTX) in a concentration of 250 ng/ml and the following experiments were performed as described.

Western blot analysis

BMM (24-well plates) were treated with LPS (1 μg/ml) or a combination of LPS (1 μg/ml) plus ANP (10^{-8}–10^{-6} M) in the presence or absence of the NPR-A antagonist HS-142-1 (100 μg/ml) for 12 h. Cells were washed with ice-cold PBS and stored at −70 C. Western blot analysis was performed according to Ref. 19, using a lysis buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 2% mercaptoethanol, 10% glycerol, 0.004% bromophenol blue), which was supplemented with a protease inhibitor cocktail (Complete). Samples were loaded on an SDS-PAGE gel (10%) and electroblotted, and COX-2 protein was detected using an anti-COX-2 monoclonal antibody and the ECL detection system. Signal intensities were evaluated by densitometric analysis (E.A.S.Y. plus system; Herolab, Wiesloch, Germany).

Detection of COX-2 mRNA

BMM were stimulated with or without LPS (1 μg/ml) in the presence or absence of ANP (10^{-6} M) or dexamethasone (10^{-5} M) for 6 h (24-well plates). RNA was prepared using TRIzol reagent or RNeasy RNA isolation kit (QIAGEN, Hilden, Germany) and pooled from six wells. Northern blot analysis with total RNA (15 μg) was performed as described previously (36). Membranes were hybridized to ^{32}P-labeled cRNA probes (2×10^6 cpm/ml). The COX-2 probe was an *Eco*RI linearized murine COX-2 cDNA in a pGEM-7 vector (from Dr. Herschman, Department of Biological Chemistry, University of California, Los Angeles). The COX-2 probe was labeled with [α-^{32}P]-UTP (50 μCi, Amersham Pharmacia Biotech) and T7 RNA polymerase. Signal intensities were evaluated by densitometric analysis (Herolab E.A.S.Y. plus system). To control for the amounts of intact mRNA, membranes were rehybridized with a ^{32}P-labeled β-actin probe. RT-PCR experiments were performed with primers for COX-2 5′-TTC AAA AGA AGT GCT GGA AAA GGT-3′ (sense) and 5′-GAT CAT CTC TAC CTG AGT GTC TTT-3′ (antisense) and β-actin 5′-CCTGACCCTGAAGTACCCCA-3′ (sense) and 5′-CGTCATGCAGCTCATAGCTC-3′ (antisense), followed by gel electrophoresis and ethidium bromide staining.

Measurement of thromboxane B_2 (TxB_2) production in vivo

Male BALB/c mice, 7–9 wk of age, from the breeding facility of the University of Konstanz, were kept at 24 C, 55% humidity, 12-h day-night rhythm, on a diet of Altromin C 1310 (Altromin Co., Lage, Germany). Mice were injected iv with ANP (0.5, 5, or 50 μg/kg body weight) or cANF (5 μg/kg body weight) diluted in 0.9% NaCl (Braun, Melsungen, Germany) with 0.1% wt/vol human serum albumin (NaCl/HSA) 15 min before administration of LPS. Control animals received the same volume of NaCl/HSA. At 0 min, 300 μg LPS (*Salmonella abortus equi*, Bioclot, Aidenbach, Germany) per kilogram body weight were injected iv. At 30 min, blood was obtained from the tail vein, for determination of TxB_2. Plasma TxB_2 was determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). All animals received humane care in accordance with the NIH guidelines and the legal requirements in Germany.

Statistical analysis

All experiments were performed at least three times. Data are expressed ± SEM. Values with $P < 0.05$ were considered significantly

different, compared with 100% (LPS-treated cells/animals only) by one sample *t* test.

Results

ANP inhibits PGE_2 production in BMM

Murine bone marrow-derived macrophages were stimulated with LPS (1 μg/ml) for 20 h to evoke PGE_2 synthesis. PGE_2, as the most important inflammatory product of COX-2 activation, was quantified in the supernatant by ELISA. Coincubation of BMM with ANP (10^{-8}–10^{-6} M) and LPS (1 μg/ml) resulted in a dose-related, significant reduction of PGE_2 production, up to 70% (Fig. 1). ANP (10^{-6} M), in the absence of LPS, did not alter the basal PGE_2 secretion.

Receptor selectivity of the ANP effect on PGE_2 production

To determine which NP-receptor mediates the inhibitory effect of ANP on PGE_2 synthesis, the following experiments were performed with LPS-stimulated cells: An antagonist of the guanylate cyclase-coupled NPR-A, HS-142-1 (37), was employed in concentrations as high as 100 μg/ml HS-142-1, known to abrogate cGMP-mediated effects of ANP in the same cell system (18, 19, 23). HS-142-1 was not able to significantly abrogate the effect of ANP on PGE_2 production when differences between the LPS+HS group and the LPS+HS+ANP group were calculated (Fig. 2A). However, statistical analysis showed that, in contrast to the LPS+ANP group, LPS+HS+ANP was not statistically different from LPS only. This suggests a certain potency of HS-142-1 to abrogate the PGE_2 inhibitory action of ANP.

The stable analog of cGMP, dibutyryl-cGMP, did not significantly affect LPS-induced PGE_2 production (Fig. 2B). Interestingly, the specific NPR-C ligand, cANF, significantly decreased LPS-induced PGE_2 secretion at a concentration of 10^{-8}–10^{-6} M without alteration of basal PGE_2 production (Fig. 2B). These results led us to suggest that the inhibition of PGE_2 production was mediated via the NPR-C.

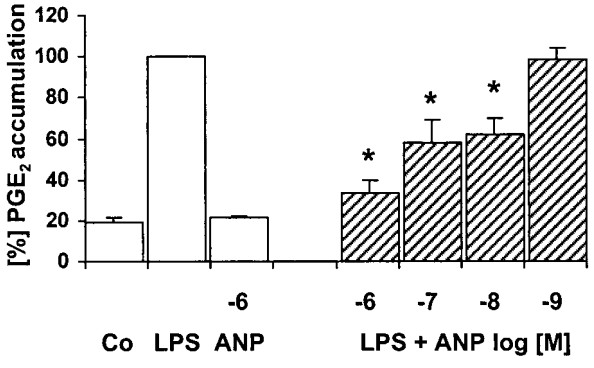

FIG. 1. ANP inhibits the formation of PGE_2 in BMM. Cells were cultured for 20 h in either medium alone [control (Co)], in medium containing ANP (10^{-6} M), and in medium containing LPS (1 μg/ml) or a combination of LPS (1 μg/ml) and various concentrations of ANP (10^{-9}–10^{-6} M). Culture supernatants were assayed for PGE_2 production using a commercially available PGE_2 ELISA (see *Materials and Methods*). Data are expressed as percentage of PGE_2 concentration accumulated in the supernatant of LPS-activated macrophages (100%) and represent the mean ± SEM of at least three independent experiments performed in triplicates. *, $P < 0.05$ represents significant differences, compared with the values seen in LPS-activated cells (one sample *t* test).

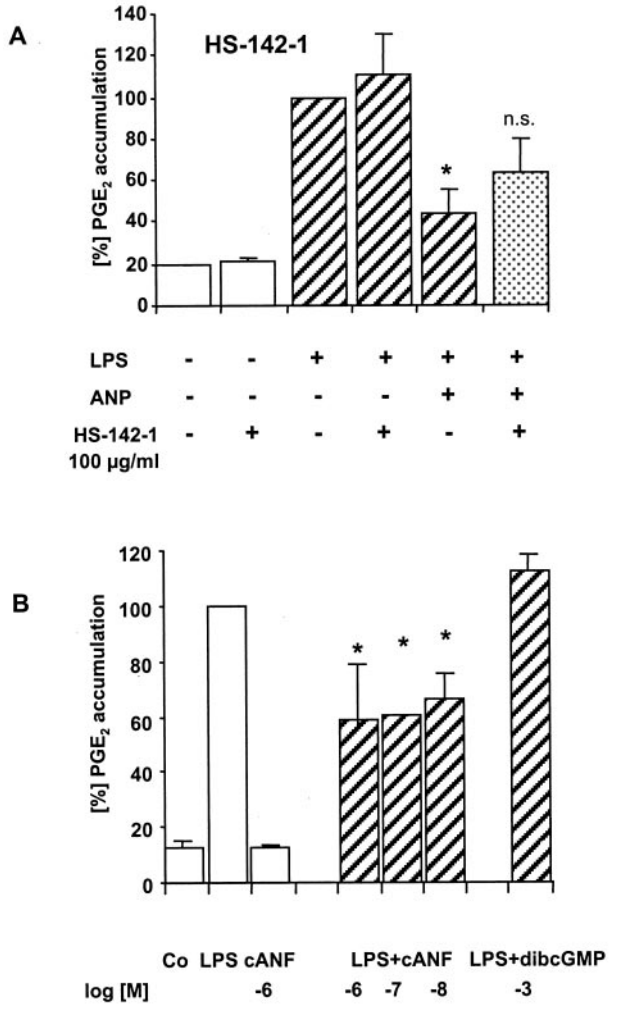

FIG. 2. Characterization of the NPR responsible for the ANP-induced PGE_2-inhibition in BMM. Cells were treated with LPS (1 μg/ml, 4 h); PGE_2 production was measured (ELISA) and referred to as 100%. A, Effect of the NPR-A antagonist, HS-142-1 (100 μg/ml), with and without ANP (10^{-6} M) added simultaneously with LPS. Results are expressed as percentage of PGE_2 accumulation in the supernatant of LPS-treated cells and represent the means ± SEM of three independent experiments performed in triplicate. n.s., Not significantly different from LPS+ANP; *, $P < 0.05$ represents significant differences, compared with the values seen in LPS-activated cells (one sample *t* test). B, Effect of the NPR-C-specific ligand, cANF (10^{-8}–10^{-6} M), and of dibutyryl-GMP (dibcGMP) at 10^{-3} M added simultaneously with LPS. Means ± SEM of at least three independent experiments performed in triplicate are shown. *, $P < 0.05$ represents significant differences, compared with the values seen in LPS-activated cells (one sample *t* test).

Effect of CNP on PGE_2 synthesis of BMM

To elucidate whether the PGE_2-inhibitory effect is specific for the binding of ANP to the NPR-C, we examined CNP, which also binds to the NPR-C. As shown in Fig. 3, CNP (in a concentration of 10^{-6} M) slightly, but significantly, inhibited PGE_2 formation of LPS-activated BMM. CNP had no effect on basal PGE_2 production (Fig. 3).

Inhibition of cAMP accumulation

The shown experiments suggested an NPR-C-mediated effect of the NP on PGE_2 production. Because several bio-

logical effects of NP exerted via the NPR-C have been shown to be related to the inhibition of adenylate cyclase (3, 7), the effect of ANP and its analogs on the accumulation of cAMP was determined in LPS-activated BMM. As shown in Fig. 4, treatment of LPS-activated BMM with ANP (10^{-7} and 10^{-6} M) and CNP (10^{-6} M) for 30 min led to a significant inhibition of cAMP accumulation, compared with controls (LPS treatment only). The specific NPR-C ligand cANF (10^{-6} M) was able to mimic this effect (Fig. 4A). When cells were pretreated overnight with PTX (250 ng/ml), effects were even more pronounced, as can be observed in Fig. 4B.

ANP treatment reduced COX-2 protein levels

To determine the mechanism by which ANP reduces LPS-induced PGE_2 production, we were interested in whether ANP influences the LPS-induced expression of COX-2 protein. Western blot experiments showed the induction of COX-2 protein in BMM after 12 h of treatment with LPS (Fig. 5). This expression was attenuated in cells cotreated with ANP. The inhibitory action of ANP on COX-2 protein was dose-related (Fig. 5A). The NPR-A antagonist HS-142-1 (100 μg/ml) did not abrogate this reduction in COX-2 protein expression (Fig. 5B).

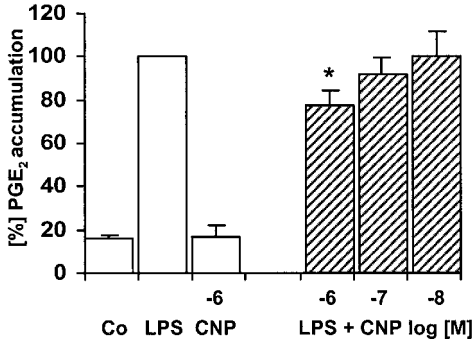

FIG. 3. CNP inhibits PGE_2 production in LPS-activated BMM. PGE_2 production in the supernatant of either cells treated with LPS (1 μg/ml) or CNP (10^{-6} M), as well as of cells costimulated with LPS and CNP is shown (untreated cells, Co). CNP was coincubated with LPS at concentrations of 10^{-8}–10^{-6} M. Results are expressed as percentage of PGE_2 accumulation in the supernatant of LPS-treated cells and represent the means ± SEM of at least three independent experiments performed in triplicate. *, $P < 0.05$ represents significant differences, compared with the values seen in LPS-activated cells (one sample *t* test).

ANP-treatment reduces COX-2 mRNA levels

To investigate whether reduction in COX-2 protein exerted by ANP is attributable to a reduction in mRNA levels, Northern blot analysis was performed. BMM were activated with LPS (1 μg/ml) in the presence or absence of ANP (10^{-6} M) or dexamethasone (10^{-5} M), and mRNA was isolated after 6 h. In unstimulated cells, no COX-2 mRNA was detectable (Fig. 6). ANP (10^{-6} M) caused a marked reduction of LPS-induced COX-2 mRNA steady-state levels. Dexamethasone (10^{-5} M), a known inhibitor of COX-2 mRNA induction, completely blocked COX-2 mRNA accumulation (Fig. 6A). Rehybridization with a β-actin probe served as a control for the amounts of intact RNA. RT-PCR experiments demonstrated a dose-related inhibition of COX-2 mRNA (Fig. 6B).

ANP, but not cANF, reduces TxB_2 production in LPS-treated mice

To demonstrate the *in vivo* relevance of our findings, we investigated a potential action of ANP on the COX system in mice. Therefore, LPS-induced TxB_2 production was measured. ANP was, in fact, shown to significantly attenuate TxB_2 production in concentrations as low as 5 μg/kg body weight, whereas there was a nonsignificant increase of TxB_2 production in a concentration of 0.5 μg/kg body weight (Fig. 7). The NPR-C agonist in a concentration of 5 μg/kg body weight also increased TxB_2 production *in vivo* (Fig. 7).

Discussion

The induction of COX-2 in macrophages represents an important pathomechanism in diverse inflammatory pro-

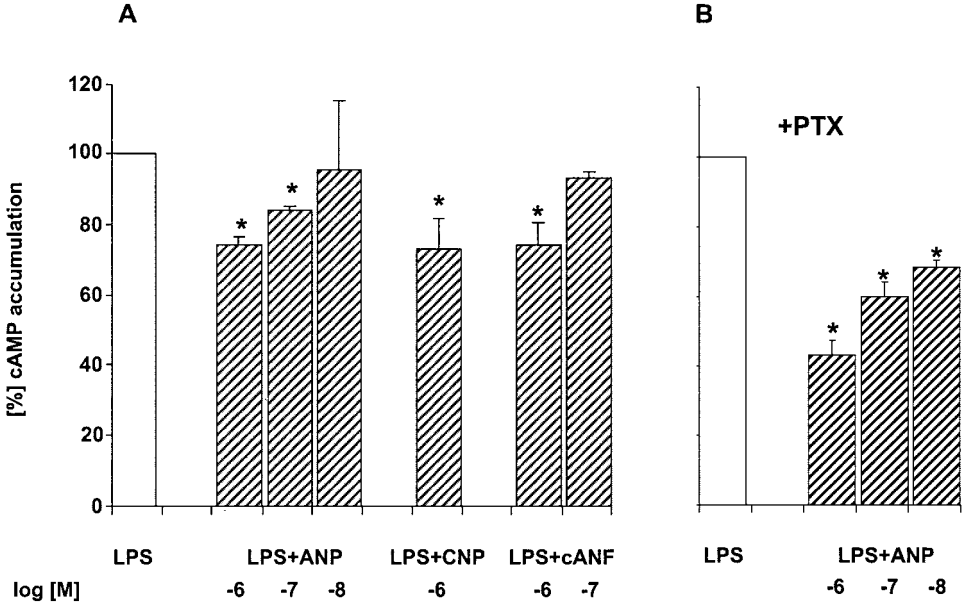

FIG. 4. NPR-C-mediated inhibition of cAMP accumulation. A, Intracellular cAMP levels of BMM were determined in cells treated with LPS (1 μg/ml) only or in cells cotreated with ANP (10^{-8}–10^{-6} M), CNP (10^{-6} M), or cANF (10^{-7} and 10^{-6} M). All cells were preincubated with 3-isobutyl-1-methylxanthine (100 μM, 10 min) in serum-free medium. cAMP levels were determined by ELISA. B, In a second set of experiments, cells were pretreated overnight with PTX in a concentration of 250 ng/ml, and experiments were performed as described above. Results are expressed as the mean ± SEM of two to three experiments performed in triplicate. *, $P < 0.05$ represents significant differences, compared with the values seen in LPS-activated cells (one sample *t* test).

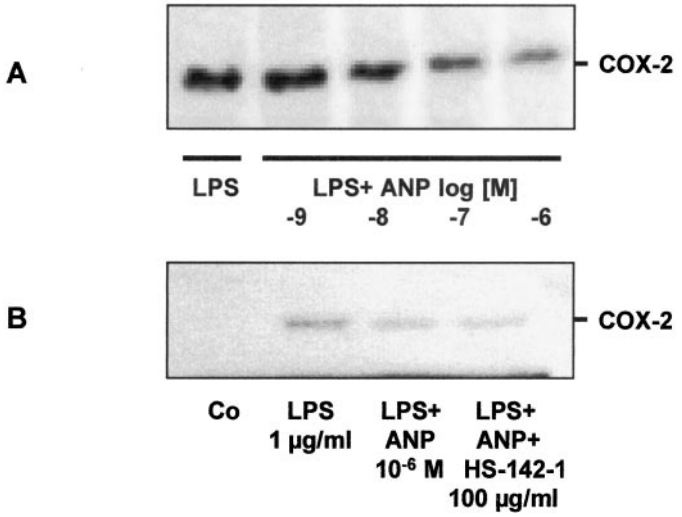

FIG. 5. NPR-C-mediated reduction of COX-2 expression. Western blot was performed from either unstimulated (Co) cells or from cells treated for 12 h with LPS (1 μg/ml), in the presence or absence of ANP (10^{-8}-10^{-6} M), with or without HS-142-1 (100 μg/ml), respectively. Blots were incubated with a monoclonal α-mouse COX-2 antibody and detected with a HRP-conjugated α-mouse antibody and the ECL system. A representative film out of each of three independent experiments is shown.

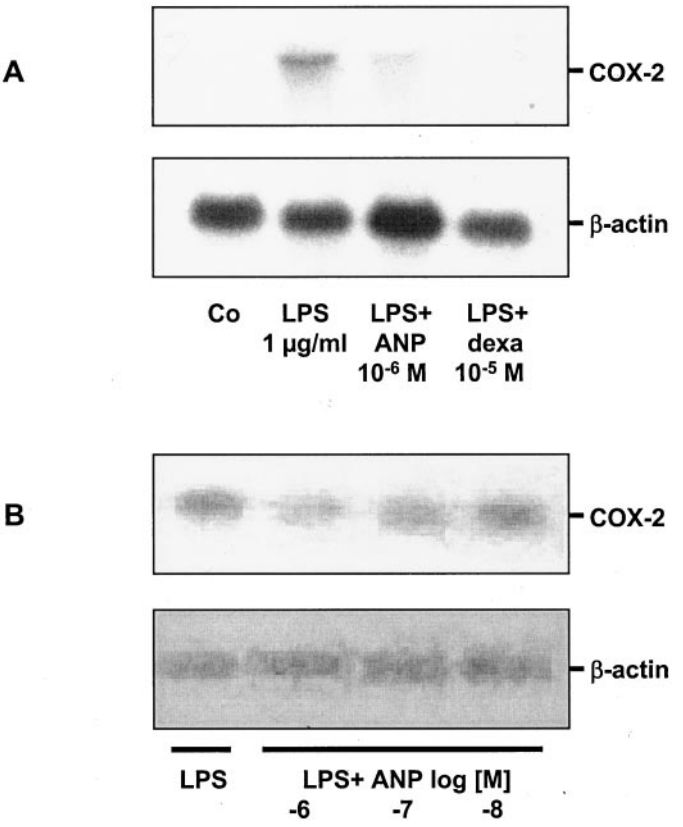

FIG. 6. ANP reduces expression of COX-2 mRNA. mRNA expression was determined in BMM, which were either unstimulated (Co) or treated with LPS (1 μg/ml) in the presence or absence of ANP (10^{-8}–10^{-6} M) and dexamethasone (dexa, 10^{-5} M), respectively, for 6 h. β-Actin served as housekeeping gene. Data show one representative out of each of three independent experiments.

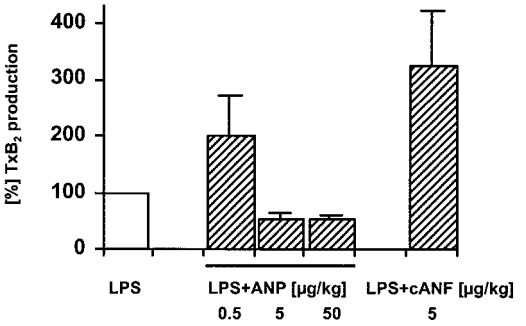

FIG. 7. Inhibition of TxB_2-production in mice. Male BALB/c mice were injected iv with ANP (0.5, 5, or 50 μg/kg body weight) or cANF (5 μg/kg body weight) with NaCl/HSA at −15 min. Control animals received the same volume of NaCl/HSA. At 0 min, 300 μg LPS per kg body weight were injected iv. At 30 min, blood was obtained from the tail vein for determination of TxB_2, which was determined by enzyme immunoassay. Data represent mean ± SEM of 4–14 animals. *, $P < 0.05$ represents significant differences, compared with LPS treatment only.

cesses (24, 25, 27). Therefore, special interest focuses on the regulatory mechanism of COX-2 expression and on tools for potential pharmacological intervention (29, 31, 32). By characterization of a novel endogenous substance, *i.e.* ANP, regulating COX-2 expression, this study might therefore be of special interest. Our data show, for the first time, that the cardiovascular hormone ANP influences COX-2 *in vitro* as well as *in vivo*. In murine macrophages: 1) ANP was shown to inhibit LPS-induced PGE_2 release. 2) In this context, evidence is provided that the NP clearance receptor contributes to this effect. 3) Evidence shows that the effect correlates to the inhibition of cAMP production. 4) Concentrating on mechanistic aspects of the inhibition exerted by ANP, ANP is suggested to regulate COX-2 at the expressional level, because ANP reduced both COX-2 mRNA and protein expression.

Our findings suggest that the NPR-C is responsible for the inhibition of COX-2 by ANP; the microbial polysaccharide HS-142-1, which selectively blocks the guanylate cyclase-linked NP receptor and cGMP production (37), was only partially, but not significantly, able to reverse the ANP effect. Employment of dibutyryl-cGMP showed no effect on PGE_2 production. Moreover, the specific ligand for the NP clearance receptor, cANF, mimicked the inhibitory action of ANP although the effect was not dose related. These data lead us to suggest that NPR-C contributes to the inhibitory action of ANP on COX-2. Additionally, a role for the NPR-A, albeit only partial, is suggested as well.

Most of the biological effects of NP are mediated via the two guanylate-cyclase-coupled receptors, the NPR-A and the NPR-B (6), including the previously observed immunomodulatory effects of ANP (15, 18, 19, 21). However, an increasing number of evidence supports the hypothesis of a biological role for the clearance receptor, beyond the capture of NP from the circulation (7). ANP was shown to exert a wide variety of effects via this receptor, such as the antiproliferative effect on endothelial cells (38), neuromodulatory functions (39), the activation of endothelial nitric oxide synthase in smooth muscle cells (40), and the increase of Ca^{2+} influx in renal cortical thick ascending limb cells (41).

Macrophages were demonstrated to express all three forms of NP-receptors (18), and NP may therefore affect macrophage functions by interfering with different binding sites. Indeed, ANP inhibits COX-2 predominantly via the NPR-C; whereas, as previously shown, the inhibitory effect of ANP on iNOS is mediated by the NPR-A (18, 19). Moreover, the effect of ANP on COX-2 expression represents the first data on the biological activity of the NPR-C in immune cells.

It is known that ligand binding to the NPR-C can lead to the inhibition of the adenylate cyclase/cAMP system (39, 42). In fact, our data showed the inhibition of cAMP production by ANP. Because (besides ANP) both CNP and cANF mimicked the effect, the mediation via the NPR-C is anticipated. The inhibition of cAMP in other cell systems is reported to depend on inhibitory G protein binding (39, 42). When we blocked binding of inhibitory G proteins by preincubation of the cells with PTX, however, the observed inhibition of cAMP accumulation was even stronger. This result leads us to suggest that the NPR-C-mediated inhibition of cAMP production in BMM is independent of PTX-sensitive inhibitory G proteins. Lee *et al.* (43) also demonstrated a reduction of cAMP content in human fibroblasts independent of PTX-sensitive G proteins. In adrenal glomerulosa cells, inhibition of cAMP production by ANP was shown to be only partially mediated via PTX-sensitive pathways (44). The mechanism by which NPR-C mediates cAMP inhibition in our cell system remains to be established. Because of the complexity of cAMP/G protein signaling, however, these investigations go beyond the scope of this work.

The functional role of cyclic nucleotides in regulating COX-2 is an intensely studied topic. Pang and Hoult (45) reported that analogs of both cAMP and cGMP inhibited the expression of COX-2 in J774 macrophages. In contrast, Tetsuka *et al.* (46) demonstrated an amplification of IL-1β-induced COX-2 expression in rat mesangial cells by NO and other cGMP-elevating substances. Our system of LPS-induced COX-2 in BMM is not sensitive to cGMP, as was shown by the fact that neither dibutyryl-cGMP nor HS-142-1 altered PGE_2 production and COX-2 expression.

These contradictory data seem to represent the fact that the influence of cyclic nucleotides on COX-2 expression highly depends on cell type and stimulus. Most of the published data, in fact, show a positive correlation between cAMP and COX-2/PGE_2 secretion. Phosphodiesterase inhibition, as well as application of dibutyryl-cAMP, elevates PGE_2 production and COX-2 expression (47–52).

The fact that CNP exerts an inhibitory effect on LPS-induced COX-2 is one further important finding of our study. All previously reported cGMP-mediated immunomodulatory effects of ANP have been demonstrated to be specific for ANP and not to be exerted by CNP. Therefore, this represents the first demonstration of an effect of CNP on immune cells. The inhibitory action of CNP on PGE_2 production, however, is exerted to a lesser degree, compared with ANP. The extent of cAMP inhibition, in contrast, is comparable. This observation may be explained by a different kinetic of CNP decay, compared with ANP, as previously demonstrated in macrophages (18) as well as by different affinity of ANP and CNP to their receptors in mouse BMM.

New antiinflammatory strategies concentrate on a selective inhibition of COX-2 enzyme activity without alteration of COX-1 activity. ANP, as a physiological regulator of COX-2, does not seem to affect COX-1 enzymatic activity, because it does not affect basal PGE_2 production. Furthermore, the reduction of LPS-induced PGE_2 secretion seems to be regulated at the transcriptional level, as indicated by an attenuated expression of both COX-2 protein and mRNA, and therefore is specific for the inducible form of COX.

ANP was demonstrated as a regulator of transcription of proinflammatory genes before. Reduction of iNOS, as well as TNF-α, however, was clearly regulated by cGMP (19, 22, 53). In contrast, the effect of ANP on COX-2 expression is independent of cGMP, because of the fact that HS-142-1 did not abrogate the effect of ANP on COX-2 protein. Because evidence is given that COX-2 transcription is regulated via cAMP (48, 49, 51), the attenuation of cAMP production by ANP may be involved in ANP-induced COX-2 inhibition in macrophages.

Our previously published papers report a regulatory role of ANP on the production of inflammatory mediators in different *in vitro* and *ex vivo* models (18, 19, 22, 23, 53). The investigation of potential effects of ANP given *in vivo* should provide further evidence for the (patho)physiological relevance of these observations. Indeed, ANP also *in vivo* inhibits the COX system. This was shown by the fact that ANP significantly attenuated the production of TxB_2 in LPS-treated mice. However, we could not observe a dose-related effect. Moreover, the NPR-C agonist cANF did not inhibit TxB_2 production. These conflicting data seem to reflect the completely different experimental setting in a whole animal compared with isolated macrophage cultures. The data might also reflect differences in the influence on COX-1, which is also responsible for the production of TxB_2. Although none of the NP affected COX-1 activity in cultivated macrophages, as indicated by a lack of effect on basal PGE_2 production, our findings might indicate an effect on COX-1 *in vivo*.

In summary, we could demonstrate a novel mechanism of action for the cardiovascular hormone ANP. ANP is supposed to be an important endogenous mediator regulating COX-2. This effect is mediated via the natriuretic clearance receptor and seems to involve transcriptional regulation. Modulation of COX-2 expression by ANP may have broad implications in inflammatory states, such as arthritis or sepsis, where, in fact, increased ANP plasma levels have been reported (35). The ability of ANP to inhibit TNF-α (22, 53), iNOS (18, 19), and COX-2 may represent important aspects supporting an antiinflammatory action of this cardiovascular hormone.

Acknowledgments

The excellent technical support of Ursula Rüberg, Raima Yaşar, and Brigitte Weiss is gratefully acknowledged. We are grateful to Dr. Matsuda (Mishima, Japan) for providing the polysaccharide HS-142-1. We also thank Dr. Herschman (UCLA) for the COX-2 cDNA probe.

Received May 9, 2001. Accepted November 8, 2001.

Address all correspondence and requests for reprints to: Alexandra K. Kiemer, Ph.D., Department of Pharmacy-Center of Drug Research,

Butenandtstrasse 5-13, 81377 Munich, Germany. E-mail: alexandra.kiemer@cup.uni-muenchen.de.

This work was supported by the "Bayerischer Habilitationsförderpreis" (to A.K.K.) and the Deutsche Forschungsgemeinschaft Vo 376/8-2.

References

1. **Rosenzweig A, Seidman CE** 1991 Atrial natriuretic factor and related peptide hormones. Annu Rev Biochem 60:229–255
2. **Samson WK** 1990 Cardiac hormones and neuroendocrine function. Adv Exp Med Biol 274:177–190
3. **Levin ER, Gardner DG, Samson WK** 1998 Natriuretic peptides. N Engl J Med 339:321–328
4. **Bovy PR** 1990 Structure activity in the atrial natriuretic peptide (ANP) family. Med Res Rev 10:115–142
5. **Garbers DL** 1992 Guanylyl cyclase receptors and their endocrine, paracrine, and autocrine ligands. Cell 71:1–4
6. **Maack T** 1996 Role of atrial natriuretic factor in volume control. Kidney Int 49:1732–1737
7. **Levin ER** 1993 Natriuretic peptide C-receptor: more than a clearance receptor. Am J Physiol 264:E483–E489
8. **Sudoh T, Minamino N, Kangawa K, Matsuo H** 1990 C-type natriuretic peptide (CNP): a new member of natriuretic peptide family identified in porcine brain. Biochem Biophys Res Commun 168:863–870
9. **Nazario B, Hu RM, Pedram A, Prins B, Levin ER** 1995 Atrial and brain natriuretic peptides stimulate the production and secretion of C-type natriuretic peptide from bovine aortic endothelial cells. J Clin Invest 95:1151–1157
10. **Suga S, Itoh H, Komatsu Y, Ogawa Y, Hama N, Yoshimasa T, Nakao K** 1993 Cytokine-induced C-type natriuretic peptide (CNP) secretion from vascular endothelial cells—evidence for CNP as a novel autocrine/paracrine regulator from endothelial cells. Endocrinology 133:3038–3041
11. **Ishizaka Y, Kangawa K, Minamino N, Ishii K, Takano S, Eto T, Matsuo H** 1992 Isolation and identification of C-type natriuretic peptide in human monocytic cell line, THP-1. Biochem Biophys Res Commun 189:697–704
12. **Vollmar AM, Schulz R** 1995 Expression and differential regulation of natriuretic peptides in mouse macrophages. J Clin Invest 95:2442–2450
13. **Chen HH, Burnett JC** 1998 C-type natriuretic peptide: the endothelial component of the natriuretic peptide system. J Cardiovasc Pharmacol 32:S22–S28
14. **Gutkowska J, Nemer M** 1989 Structure, expression, and function of atrial natriuretic factor in extraatrial tissues. Endocr Rev 10:519–536
15. **Vollmar AM** 1996 Natriuretic peptides and immune function. In: Samson WK, Levin ER, eds. Natriuretic peptides in health and disease. Clifton, NJ: Humana; 275–288
16. **Vollmar AM, Lang RE, Hanze J, Schulz R** 1990 The rat thymus—a site of atrial natriuretic peptide synthesis. Peptides 11:33–37
17. **Vollmar AM, Schmidt KN, Schulz R** 1996 Natriuretic peptide receptors on rat thymocytes: inhibition of proliferation by atrial natriuretic peptide. Endocrinology 137:1706–1713
18. **Kiemer AK, Vollmar AM** 1997 Effects of different natriuretic peptides on nitric oxide synthesis in macrophages. Endocrinology 138:4282–4290
19. **Kiemer AK, Vollmar AM** 1998 Autocrine regulation of inducible nitric-oxide synthase in macrophages by atrial natriuretic peptide. J Biol Chem 273:13444–13451
20. **Vollmar AM** 1997 Influence of atrial natriuretic peptide on thymocyte development in fetal thymic organ culture. J Neuroimmunol 78:90–96
21. **Vollmar AM, Förster R, Schulz R** 1997 Effects of atrial natriuretic peptide on phagocytosis and respiratory burst in murine macrophages. Eur J Pharmacol 319:279–285
22. **Kiemer AK, Hartung T, Vollmar AM** 2000 cGMP-mediated inhibition of TNF-α production by the atrial natriuretic peptide in murine macrophages. J Immunol 165:175–181
23. **Kiemer AK, Vollmar AM** 2001 Elevation of intracellular calcium levels contributes to the inhibition of inducible nitric oxide synthase by atrial natriuretic peptide. Immunol Cell Biol 79:11–17
24. **Williams CS, Mann M, DuBois RN** 1999 The role of cyclooxygenases in inflammation, cancer, and development. Oncogene 18:7908–7916
25. **Lipsky PE, Isakson PC** 1997 Outcome of specific COX-2 inhibition in rheumatoid arthritis. J Rheumatol 24:9–14
26. **Herschman HR** 1994 Regulation of prostaglandin synthase-1 and prostaglandin synthase-2. Cancer Metastasis Rev 13:241–256
27. **Smith WL, Garavito RM, DeWitt DL** 1996 Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J Biol Chem 271:33157–33160
28. **Kujubu DA, Fletcher BS, Varnum BC, Lim RW, Herschman HR** 1991 TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J Biol Chem 266:12866–12872
29. **Masferrer JL, Zweifel BS, Seibert K, Needleman P** 1990 Selective regulation of cellular cyclooxygenase by dexamethasone and endotoxin in mice. J Clin Invest 86:1375–1379
30. **Inoue H, Yokoyama C, Hara S, Tone Y, Tanabe T** 1995 Transcriptional regulation of human prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. Involvement of both nuclear factor for interleukin-6 expression site and cAMP response element. J Biol Chem 270:24965–24971
31. **DuBois RN, Awad J, Morrow J, Roberts LJ, Bishop PR** 1994 Regulation of eicosanoid production and mitogenesis in rat intestinal epithelial cells by transforming growth factor-α and phorbol ester. J Clin Invest 93:493–498
32. **Crofford LJ, Wilder RL, Ristimaki AP, Sano H, Remmers EF, Epps HR, Hla T** 1994 Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues. Effects of interleukin-1β, phorbol ester, and corticosteroids. J Clin Invest 93:1095–1101
33. **Subbaramaiah K, Telang N, Ramonetti JT, Araki R, DeVito B, Weksler BB, Dannenberg AJ** 1996 Transcription of cyclooxygenase-2 is enhanced in transformed mammary epithelial cells. Cancer Res 56:4424–4429
34. **Aiura K, Ueda M, Endo M, Kitajima M** 1995 Circulating concentrations and physiologic role of atrial natriuretic peptide during endotoxic shock in the rat. Crit Care Med 23:1898–1906
35. **Forslund T, Hannonen P, Reitamo S, Fyhrquist F** 1995 Hypertension in cyclosporin A-treated patients is independent of circulating endothelin levels. J Intern Med 238:71–75
36. **Vollmar AM, Schulz R** 1994 Gene expression and secretion of atrial natriuretic peptide by murine macrophages. J Clin Invest 94:539–545
37. **Morishita Y, Sano T, Ando K, Saitoh Y, Kase H, Yamada K, Matsuda Y** 1991 Microbial polysaccharide, HS-142-1, competitively and selectively inhibits ANP binding to its guanylyl cyclase-containing receptor. Biochem Biophys Res Commun 176:949–957
38. **Pedram A, Razandi M, Hu RM, Levin ER** 1997 Vasoactive peptides modulate vascular endothelial cell growth factor production and endothelial cell proliferation and invasion. J Biol Chem 272:17097–17103
39. **Drewett JG, Ziegler RJ, Trachte GJ** 1990 Neuromodulatory effects of atrial natriuretic factor are independent of guanylate cyclase in adrenergic neuronal pheochromocytoma cells. J Pharmacol Exp Ther 255:497–503
40. **Murthy KS, Teng B, Jin J, Makhlouf GM** 1998 G protein-dependent activation of smooth muscle eNOS via natriuretic peptide clearance receptor. Am J Physiol 275:C1409–C1416
41. **Dai L-J, Quamme GA** 1993 Atrial natriuretic peptide initiates Ca^{2+} transients in isolated renal cortical thick ascending limb cells. Am J Physiol 265:F592–F597
42. **Anand-Srivastava MB, Sairam MR, Cantin M** 1990 Ring-deleted analogs of atrial natriuretic factor inhibit adenylate cyclase/cAMP system. Possible coupling of clearance atrial natriuretic factor receptors to adenylate cyclase/cAMP signal transduction system. J Biol Chem 265:8566–8572
43. **Lee MA, West RE, Moss J** 1988 Atrial natriuretic factor reduces cyclic adenosine monophosphate content of human fibroblasts by enhancing phosphodiesterase activity. J Clin Invest 82:388–393
44. **MacFarland RT, Zelus BD, Beavo JA** 1991 High concentrations of a cGMP-stimulated phosphodiesterase mediate ANP-induced decreases in cAMP and steroidogenesis in adrenal glomerulosa cells. J Biol Chem 266:136–142
45. **Pang L, Hoult JR** 1997 Repression of inducible nitric oxide synthase and cyclooxygenase-2 by prostaglandin E2 and other cyclic AMP stimulants in J774 macrophages. Biochem Pharmacol 53:493–500
46. **Tetsuka T, Daphna-Iken D, Miller BW, Guan Z, Baier LD, Morrison AR** 1996 Nitric oxide amplifies interleukin 1-induced cyclooxygenase-2 expression in rat mesangial cells. J Clin Invest 97:2051–2056
47. **Minghetti L, Polazzi E, Nicolini A, Creminon C, Levi G** 1997 Up-regulation of cyclooxygenase-2 expression in cultured microglia by prostaglandin E2, cyclic AMP and non-steroidal anti-inflammatory drugs. Eur J Neurosci 9:934–940
48. **Fiebich BL, Biber K, Lieb K, van Calker D, Berger M, Bauer J, Gebicke-Haerter PJ** 1996 Cyclooxygenase-2 expression in rat microglia is induced by adenosine A2a-receptors. Glia 18:152–160
49. **Lo CJ, Fu M, Lo FR, Cryer HG** 2000 Cyclooxygenase 2 (COX-2) gene activation is regulated by cyclic adenosine monophosphate. Shock 13:41–45
50. **Klein T, Ullrich V, Pfeilschifter J, Nusing R** 1998 On the induction of cyclooxygenase-2, inducible nitric oxide synthase and soluble phospholipase A2 in rat mesangial cells by a nonsteroidal anti-inflammatory drug: the role of cyclic AMP. Mol Pharmacol 53:385–391
51. **Zhou XL, Lei ZM, Rao CV** 1999 Treatment of human endometrial gland epithelial cells with chorionic gonadotropin/luteinizing hormone increases the expression of the cyclooxygenase-2 gene. J Clin Endocrinol Metab 84:3364–3377
52. **Juergens UR, Degenhardt V, Stober M, Vetter H** 1999 New insights in the bronchodilatory and anti-inflammatory mechanisms of action of theophylline. Arzneimittelforschung 49:694–698
53. **Kiemer AK, Vollmar AM, Bilzer M, Gerwig T, Gerbes AL** 2000 Atrial natriuretic peptide reduces expression of TNF-α mRNA during reperfusion of the rat liver upon decreased activation of NF-κB and AP-1. J Hepatol 33:236–246