Annexin A1 modulates macula densa function by inhibiting cyclooxygenase 2

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Annexin A1 (ANXA1) exerts anti-inflammatory effects through multiple mechanisms including inhibition of prostaglandin synthesis. Once secreted, ANXA1 can bind to G protein-coupled formyl peptide receptors (Fpr) and activate diverse cellular signaling pathways. ANXA1 is known to be expressed in cells of the juxtaglomerular apparatus, but its relation to the expression of cyclooxygenase 2 (COX-2) in thick ascending limb and macula densa cells has not been elucidated. We hypothesized that ANXA1 regulates the biosynthesis of COX-2. ANXA1 abundance in rat kidney macula densa was extensively co-localized with COX-2 (95%). Furosemide, an established stimulus for COX-2 induction, caused enhanced expression of both ANXA1 and COX-2 with maintained co-localization (99%). In ANXA1-deficient mice, COX-2-positive cells were more numerous than in control mice (+107%; normalized to glomerular number; p<0.05) and renin expression was increased (+566%; normalized to glomerular number; p<0.05). Cultured macula densa cells transfected with full-length rat ANXA1 revealed downregulation of COX-2 mRNA (-59%; P<0.05). Similarly, treatment with dexamethasone suppressed COX-2 mRNA in the cells (-49%; p<0.05), while inducing ANXA1 mRNA (+56%; P<0.05) and ANXA1 protein secretion. Inhibition of the ANXA-1 receptor Fpr1 with cyclosporin H blunted the effect of dexamethasone on COX-2 expression. These data show that ANXA1 exerts an inhibitory effect on COX-2 expression in the macula densa. ANXA1 may be a novel intrinsic modulator of renal juxtaglomerular regulation by inhibition of PGE2 synthesis.

KEYWORDS
Annexin A1, juxtaglomerular apparatus, prostaglandins, glucocorticoid, formyl-peptide receptors

**INTRODUCTION**

Macula densa (MD)-derived prostaglandin (PG) E2 is an important paracrine mediator of tubulo-vascular crosstalk at the level of the renal juxtaglomerular apparatus (JGA). Specifically, PGE2 is released by MD cells in response to a reduction of luminal NaCl concentration, and it stimulates renin synthesis and release from the afferent arteriolar granular cells (22). Furthermore, PGE2 maintains glomerular filtration rate (GFR) in the setting of compromised renal perfusion (23, 41, 42) through direct and indirect dilatation of the afferent arteriole (18). PGE2 synthesis in MD and adjacent thick-ascending limb (TAL) requires the coordinated action of several enzymes including phospholipase A2 group IVA (PLA2G4) and cyclooxygenase type 2 (COX-2) as rate-determining catalyzers of the local reaction cascade (5, 17, 28). Previous studies have documented COX-2 upregulation in the setting of low renal perfusion pressure, reduced dietary salt intake, and NaCl transport blockade by furosemide (17, 40, 46). Conversely, high salt intake, high circulating levels of angiotensin (Ang) II, and increased renal perfusion pressure reduce COX-2 abundance (16, 40). Glucocorticoids and mineralocorticoids also effectively inhibit COX-2 (27, 48). To date, the mechanisms involved in the control of juxtaglomerular COX-2 synthesis are imperfectly defined.

Previous experimental evidence indicates that intrinsic proteins participate in the regulation of prostaglandin synthesis during inflammation. Of particular interest in this regard is annexin A1 (ANXA1), a calcium-dependent phospholipid-binding protein that mediates glucocorticoid action, reduces cytosolic phospholipase A2 by reducing PLA2 gene expression, and limits COX-2 abundance (11, 20, 31). ANXA1 is secreted in...
response to glucocorticoids in a cell-specific process involving its phosphorylation and excretion by the ATP-binding cassette transporter ABCA1 (10, 38, 44). Once externalized, ANXA1 or its N-terminal peptides may bind to formyl peptide receptors (FPR) (9, 19, 36, 43). In humans, three different FPRs with distinct expression profiles and pharmacological properties have been identified. Designated as FPR1, FPR2 (formerly termed as FPRL1), and FPR3 (formerly termed as FPRL2), these receptors interact with a variety of exogenous or endogenous ligands (30, 47). FPRs are activated by bacterial peptides with an N-terminal, formylated methionine. N-formyl-methionyl-leucyl-phenylalanine (fMLF) has been widely used to study the biological effects of FPRs (47). The cyclic undecapeptide cyclosporin H (CsH) is a potent FPR1 inhibitor (45), whereas the peptide, WRW4, is a FPR2 and FPR3 antagonist (4, 37). ANXA1 and its peptides bind to all human FPRs. The responses are cell-specific and depend on the concentration of the ligand (30, 47). Eight members of the Fpr family have been reported in mice; however, only Fpr1, Fpr2 (formerly termed Fpr-rs2), and Fpr3 (formerly termed Fpr-rs1) have been studied in detail (47). Based on sequence homology and functional analysis Fpr1 is considered to be homologue to the human FPR1 while both Fpr2 and Fpr3 share characteristics of the human FPR2 (47).

ANXA1 is expressed in the macula densa segment of rodents (29), but its local effects are unexplored. We studied the regulation of macula densa ANXA1 expression and its potential role in regulating local COX-2 activity. We show that ANXA1 expression is regulated by Na⁺-K⁺-2Cl⁻-cotransporter-dependent NaCl transport. We suggest that the inhibitory effect of glucocorticoids on macula densa COX-2 expression is mediated by ANXA1, probably by activating Fpr1.
MATERIALS AND METHODS

Materials. All chemicals and pharmacological substances were obtained from Sigma Aldrich (Sigma Aldrich, Munich, Germany) unless indicated otherwise. Cell culture media and serum were from Pan Biotech (Aidenbach, Germany). Antibody against ANXA1 (rabbit anti human ANXA1 antibody; catalog nr. LS-C20640) was from Lifespan Biosciences (Cupertino, USA). Antibody against COX-2 (rabbit anti murine COX-2 antibody; item number 160126) was from Cayman Chemicals (Ann Arbor, USA). Antibody against PLA2G4 (rabbit anti human PLA2G4 antibody; code: ab58375) was from Abcam (Cambridge, UK), and antibody against the neuronal isoform of nitric oxide synthase (mouse monoclonal anti-rat NOS1 antibody; product number: N 2280) was from SIGMA (Saint Louis, USA).

Animal studies and tissue preservation. All animal studies were performed according to NIH guidelines and were approved by the Berlin council on animal care (permission number G006-02/05). Male adult Sprague Dawley rats (SD rats; \( n = 8 \)) were obtained from Charles River (Sulzfeld, Germany) and kept in the animal facility of the Charité. After an acclimatization period of one week animals were randomly divided into 2 groups (\( n = 4 \) per group) and treated for 3 days with furosemide (Furo; 12mg/d; Sigma Aldrich, Germany) or vehicle via osmotic minipump (ALZET minipump model 2ML1, Charles River, Sulzfeld, Germany). To avoid excessive volume depletion animals received 0.45% NaCl solution as drinking fluid. At the end of the treatment period animals were processed for histological studies as previously described (32, 35). Briefly, animals were anesthetized by isoflurane inhalation (Abbott, Wiesbaden, Germany) followed by an injection of pentobarbital sodium (0.06 mg/g body wt ip, FAGRON, Barsbüttel,
Germany). The abdominal cavity was opened and the abdominal aorta was cannulated using a Teflon tube (KRONLAB, Dinslaken, Germany). Kidneys were perfusion-fixed with 3% paraformaldehyde (PFA, Merck, Darmstadt, Germany) in PBS via retrograde perfusion of the abdominal aorta. After perfusion, kidneys were carefully removed and processed for paraffin embedding or cryostat sectioning.

ANXA1 knockout mice were originally generated by Hannon et al. and kept on a C57Bl/6 genetic background. Heterozygous animals were bred in the local animal facility and the offspring was genotyped as previously described (15). At three month of age kidneys of male knockout and wild type mice (n=4 for each genotype) were perfusion-fixed as described above and processed for histological studies.

Cell culture studies. Cell culture experiments were performed using a mouse macula densa cell line (MMDD1) generated by Yang et al. (46). For initial characterization studies cells were grown to subconfluence in DMEM supplemented with 10% fetal calf serum and antibiotics as previously described (46). Prior to use cells were serum starved for 24h and subsequently fixed in 3% PFA solution or harvested and processed for cDNA generation. Expression of ANXA1, COX-2, Fpr1, Fpr2, Fpr3 and ABCA1 mRNA was verified by conventional PCR using specific primers (table 1). Intracellular localization of ANXA1, PLA2G4 and COX-2 was determined by immunofluorescence.

Experimental protocols. All cell culture studies were run in triplicates and repeated at least 3 times in independent experiments. To study the effects of glucocorticoids on ANXA1 and COX-2 expression MMDD1 cells were treated for 8 h with 0.1 µmol dexamethasone (Dex; Sigma Aldrich, Munich, Germany) dissolved in 100% ethanol. The
effect of Dex on ANXA1 release by MMDD1 cells was determined after 4h. Vehicle
treated cells served as controls. To avoid toxic effects of the solvent final ethanol
ccentration was kept below 1%o. At the end of the treatment period cells were
harvested and processed for cDNA generation or Western blot analysis. Cell culture
supernatant was collected in parallel and subjected to trichloroacetic acid precipitation
using routine methodology. Briefly, supernatants were centrifuged at 4°C for 10 min at
800x g to remove detached cells and cell detritus. One ml of the supernatant was
supplemented with 250µl of a 100% (w/v) solution of trichloroacetic acid in distilled
water. Supernatants were incubated for 10 min at 4°C and subsequently centrifuged at
18,000x g. The precipitated proteins were washed twice with ice-cold acetone, air dried
and resuspended in 2x Laemmli buffer. The ANXA1 content of the cells and the
supernatant was determined by Western blot. Beta actin content of the cell
homogenates was measured in parallel to ensure equal loading.

Extracellular ANXA1 has been shown to activate receptors of the FPR family (Review in
(19)). To demonstrate an effect of FPRs on MMDD1 COX-2 expression cells were
treated for 8h with the nonselective FPR agonist fMLF (0.1 µM; Sigma Aldrich, Munich,
Germany) dissolved in dimethylsulfoxide (DMSO; Sigma Aldrich, Munich, Germany) or
the N-terminal ANXA1 fragment AC2-26 (50 µM; AnaSpec, Fremont, USA) dissolved in
1M NaOH solution. Vehicle-treated cells served as controls. After 8h cells were
harvested and processed for cDNA generation or Western blot analysis. To elucidate the
identity of the specific FPR involved in the dexamethasone-mediated suppression of
COX-2 mRNA we employed CsH as selective antagonist for Fpr1 (CsH; ENZO Life
Sciences, Lörrach, Germany) and WRW4 as inhibitor for Fpr2 and Fpr3 (WRW4;
AnaSpec, Fremont, USA). Cells were either treated with dexamethasone, CsH or WRW4 alone or with a combination of dexamethasone and the respective inhibitor. Vehicle-treated cells served as controls. After 8h cells were harvested and processed for cDNA generation.

For overexpression studies MMDD1 cells were transiently transfected using a commercially available full-length rat ANXA1 clone (IRBPp993H041D; ImaGenes Inc., Berlin, Germany) and FuGENE transfection reagent (Promega, Mannheim, Germany) according to the manufacturer’s instructions. After 24h cells and cell culture supernatant were harvested and processed as described above.

Conventional and real time polymerase chain reaction (PCR). Expression of ANXA1, COX-2, Fpr1, Fpr2, Fpr3 and ABCA1 mRNA in MMDD1 cells and mouse kidney extracts was verified by conventional PCR using specific primers (table 1). All DNA primers were designed using NCBI primer blast software (NCBI, Bethesda, USA). Total mRNA was isolated using the Qiagen RNeasy mini kit following the manufacturer's protocol (Qiagen, Hilden, Germany). After digestion of genomic DNA by DNase 1 treatment (Qiagen, Hilden, Germany) cDNA was generated by reverse transcription using the Applied Biosystems cDNA synthesis kit (Applied Biosystems, Darmstadt, Germany). PCR reaction without the addition of cDNA served as a negative control. All PCR products were sequenced in order to verify their identity (DLMBC sequencing service, Berlin, Germany).

Table 1. cDNA primers
<table>
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<th>Product</th>
<th>sense primer</th>
<th>antisense primer</th>
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<tr>
<td>ANXA1</td>
<td>CCCTGGATGAAACCTTGAAA</td>
<td>CATTCACGTCTGTCCCCTTT</td>
</tr>
<tr>
<td>COX-2</td>
<td>ACACTCTATCAGCTGCCATCC</td>
<td>GAAGGGGACACCCCTTTTCACAT</td>
</tr>
<tr>
<td>Fpr1</td>
<td>CCACTCTCTGTCAGAGGA</td>
<td>GCACAGTGGAACTCAAAGCA</td>
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<tr>
<td>Fpr2 (Fpr-rs2)</td>
<td>GTCAAGATCAACAGAAGAAACC</td>
<td>GGGCTCTCTCAAGACTATAAGG</td>
</tr>
<tr>
<td>Fpr3 (Fpr-rs1)</td>
<td>GGCAACTCTCTGAGGAAAGCC</td>
<td>GGCTCTCGGTAGCAGAGA</td>
</tr>
<tr>
<td>ABCA1</td>
<td>GTGACGCGCTGGCTCTGGTGTTGC</td>
<td>CCAGCCGGGCTTCCAGACCTGCTTCC</td>
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TaqMan quantitative RT PCR for ANXA1 and COX-2 was performed using the Applied Biosystems probes Rn.01454280 for ANXA1 and Mm00478374 for COX-2 and the 7500 Fast Real-Time PCR system (Applied Biosystems, Darmstadt, Germany) following the manufacturer's instructions. The mRNA levels of GAPDH were determined in parallel and served as loading control (catalog number 4352338E, Applied Biosystems, Darmstadt, Germany). Expression levels were calculated according to the $2^{-\Delta\Delta CT}$ method and expressed as x-fold of control (26).

Western blot analysis. Western blot analysis was performed as described earlier (33). Briefly, samples were homogenized in sucrose-triethanolamine buffer (33). Nuclei were removed by centrifugation at 800x g for 10 min at 4°C. Supernatant protein concentration was determined by bicinchoninic acid protein assay reagent kit (Thermo Fisher Scientific, Bonn, Germany). Samples were subsequently separated by SDS polyacrylamide gel electrophoresis in a 10% gel (50 µg protein/lane) and electrophoretically transferred to nitrocellulose membranes. Nonspecific protein binding sites were blocked by immersion in 5% non-fat dry milk in PBS. Membranes were subsequently incubated with the respective primary antibody for 1h at room temperature followed by an overnight-incubation at 4°C. Bound antibody was detected using the respective HRP-conjugated secondary antibodies (DAKO, Hamburg, Germany) and a chemiluminescence kit (GE healthcare, Munich, Germany). Developed X-ray films were
scanned and densitometrically quantified using AlphalImager software (Cell Biosciences, Santa Clara, USA). Expression levels were normalized to the expression of the housekeeping gene beta-actin using a well characterized monoclonal antibody (1:20 000, Sigma-Aldrich, Hamburg, Germany) and expressed as % of control.

**mRNA in situ hybridization.** ANXA1 and renin mRNA expression were evaluated using non-radioactive in situ hybridization. Riboprobes were generated by in vitro transcription either from the rat full-length ANXA1 cDNA-clone described above, or from a 300 bp PSTI / KPN1 fragment of rat renin cDNA subcloned in PGEM3 vector for renin (3, 14) using digoxynegenin (DIG)-labeled UTP and T7 or SP6 RNA polymerase (Roche Applied Science, Mannheim, Germany). Following transcription, ANXA1 riboprobes were subjected to alcaline hydrolysis for better tissue penetration. Hybridization was performed as previously described (34). Renin mRNA expression was quantified by counting positive renin signals normalized to the total number of glomeruli (34).

**Immunostaining.** Immunofluorescence and immunohistochemistry for ANXA1,NOS1, COX-2 and PLA2G4 were performed using established methodology (34). Briefly, 4 μm paraffin sections were deparaffinized and subjected to boiling in 0.1 M citrate buffer for antigen retrieval. For immunohistochemistry tissue peroxidases were blocked by incubation in 3% hydrogenperoxide in methanol. After repeated rinsing in PBS, sections were incubated in 0.5% Triton X-100/PBS for 30 min. Unspecific protein binding sites were blocked by a 2h incubation with 5% dry milk in PBS. Primary antibodies were applied in 5% dry milk overnight at room temperature. After being rinsed in PBS, sections were incubated with Cy2 or Cy3-labeled secondary antibodies for fluorescence-signal generation and with HRP-labeled secondary antibodies for immunohistochemistry. Nuclei were stained using 4’-6-diamidino-2-phenylindole (DAPI)
as indicated. For all antibodies, control experiments were conducted in which the primary antibody was omitted. The specificity of the ANXA1 antibody was further verified by the absence of immunostaining in the kidneys of ANXA1 knockout mice. Sections were examined using a Zeiss exciter 5 confocal microscope (Zeiss, Jena, Germany). Immunohistochemistry-images were acquired using a Leica DMRB microscope equipped with a interference contrast module (Leica, Wetzlar, Germany), a SPOT RT 2.3.0 digital camera (Diagnostic Instruments, Sterling Heights, MI, USA), and the MetaVue imaging System (Molecular Devices, Downingtown, PA USA). Immunoreactive macula densa cells with a signal-intensity above the surrounding TAL cells were counted. The number of cells with strong immunoreactivity was normalized to the total number of glomeruli on the section and the result was expressed as immunoreactive cells / 100 glomeruli. COX-2 expression was quantified as previously described (34).

**Statistical analysis.** Statistical analysis was performed using ANOVA with Tukey-Kramer post hoc test. Where only two groups were compared, Student's t test was used. Null hypothesis was excluded when $P$ was < 0.05.

**RESULTS**

*Localization of ANXA1 and COX-2 in rat kidney and cultured macula densa cells.*

Immunohistochemistry for ANXA1 in rat kidneys showed widespread distribution of immunoreactive product throughout the renal cortex with intense signal in macula densa cells. Surrounding TAL cells showed weak to intermediate signal intensity and were clearly distinguishable from the macula densa. ANXA1 signal was also detected in podocytes and Bowman capsule as well as in the medullary collecting duct and in interstitial cells in cortex and medulla. The findings correspond to an earlier report (29).
Under control conditions, ANXA1 immunofluorescence in macula densa cells was evenly distributed throughout the cytosol and less intense in the nucleus (Fig. 1A). This intracellular distribution pattern is recapitulated in MMDD1 cells (Fig. 1B).

Analysis of the intracellular COX-2 (Fig. 1 C,D) and PLA2G4 (Fig. 1E,F) distribution demonstrated a cytosolic pattern with an accumulation of immunoreactive protein in the perinuclear space of rat macula densa (Fig. 1C,E) and MMDD1 cells (Fig. 1D,F), thus confirming previous data (17, 28).

MMDD1 mRNA expression profile.

In situ hybridization for ANXA1 confirmed accumulation of ANXA1 mRNA in the macula densa of control rats (Fig. 2A). PCR analysis revealed the presence of mRNA for ANXA1, COX-2 and the ANXA1 receptors Fpr1, Fpr2 and Fpr3 in both murine kidney and MMDD1 cells (Fig. 2B). In addition, we also detected mRNA for the putative ANXA1 transporter ABCA1 in both sources. The specificity of the PCR products was confirmed by TA cloning and subsequent sequencing.

Effect of furosemide treatment on macula densa ANXA1 expression.

The effect of macula densa salt reabsorption on ANXA1 expression was tested in furosemide-treated rats. Treatment caused an increase in the number of cells with strong ANXA1 immunoreactivity compared to control animals (Fig. 3A,B). Quantification of ANXA1 immunoreactive macula densa cells revealed a significant 2.5-fold increase in the number of immunoreactive cells in the furosemide-treated animals as compared to controls (126 ± 19 vs. 50 ± 16 cells/100 glomeruli; p<0.05; Fig. 3C). Intracellular ANXA1 distribution was not affected.
Accumulation of ANXA1 in macula densa cells of furosemide treated animals was further documented by double labeling with the macula densa marker protein NOS1. Virtually all NOS1 immunoreactive macula densa cells also expressed high levels of ANXA1 both in controls and in furosemide-treated specimens alike (Fig. 4).

As established earlier (29), the number of COX-2 immunoreactive macula densa cells was significantly increased in parallel (183 ± 23.6 vs. 54 ± 8.7 cells/100 glomeruli; p<0.05). ANXA1 and COX-2 were extensively colocalized in macula densa in 95 ± 5% of all cells, and this level of co-localization was not affected by furosemide (99 ± 0.78%; p=0.2; Fig. 5).

Macula densa COX-2 expression in ANXA1 knockout mice.
ANXA1 knockout mice demonstrated absence of ANXA1 signal confirming specificity of the antibody (Fig. 6A,B). Analysis of macula densa-associated COX-2 immunoreactivity revealed a significantly higher number of COX-2 expressing cells in ANXA1 knockout mice, compared to controls (56 ± 8.4 vs. 27 ± 4.2 cells/100 glomeruli; p<0.05; Fig. 6C,D).

Juxtaglomerular renin mRNA expression in ANXA1 knockout mice.
Renin mRNA expression was determined by in situ hybridization. Quantification of the fraction of renin-positive glomeruli revealed significantly increased numbers in the ANXA1 knockout mice compared to controls (25.4 ± 6.4 vs. 3.8 ± 1.3 signals/100 glomeruli; p<0.05; Fig. 7A,B)
Effect of ANXA1 overexpression on MMDD1 COX-2 expression.

To elucidate the effect of ANXA1 on macula densa COX-2 expression MMDD1 cells were transiently transfected with an expression vector coding for rat full length ANXA1 under the control of a cytomegalovirus (CMV) promoter. The transfection caused a robust induction of ANXA1 mRNA levels (+750 ± 40%, compared to controls; p<0.05; Fig. 8A). Concomitantly, the amount of COX-2 mRNA was reduced (-59 ± 2%, compared to controls; p<0.05; Fig. 8B). To characterize the mechanism of the inhibitory effect, we tested, whether or not ANXA1 overexpression would lead to an increased release of the protein into the medium. We subjected cell culture supernatants of transfected and control cells to trichloracetic acid precipitation and subsequent Western blot analysis. This experiment revealed a significantly increased abundance of ANXA1 in the supernatant of the transfected cells as compared to controls (Fig. 8C), demonstrating an increased release of the protein. Analysis of immunoreactive products in the supernatant revealed two distinct bands which are likely to reflect full length ANXA1 and a smaller, truncated form which may result from proteolytic cleavage of the protein following its secretion (25).

Role of formyl peptide receptors for the control of MMDD1 COX-2 expression.

Extracellular ANXA1 has been shown to activate different receptors of the FPR family. Based on the observation that MMDD1 cells express Fpr1, Fpr2 and Fpr3 (Fig. 2B), we hypothesized that an activation of these receptors would affect MMDD1 COX-2 expression. To test this idea, MMDD1 cells were treated for 8h with fMLF, a well-characterized agonist of all three receptors. We observed a significant reduction in MMDD1 COX-2 mRNA (-34 ± 6% vs. controls; p<0.05; Fig. 9B) and protein abundance
Effect of Dex on MMDD1 ANXA1 expression and secretion.

We next sought to elucidate whether or not release of ANXA1 from macula densa cells was sensitive to stimuli previously shown to be effective in pituitary folliculo-stellate cells (39), monocytes (10), and other leukocytes (11). Treating MMDD1 cells for 8 h with 0.1 µM dexamethasone caused a marked increase in the amount of ANXA1 in the medium compared to vehicle-treated cells, whereas the intracellular concentration remained stable (Fig. 10A). After 8h, we also observed an accumulation of ANXA1 mRNA (+56 ± 10% of control; p<0.05; Fig. 10B). Concomitantly, the amount of COX-2 mRNA was decreased (-49 ± 10% of controls; p<0.05; Fig. 10C). Again, analysis of immunoreactive products revealed the presence of both full-length and truncated ANXA1.

Role of externalized ANXA1 or AC2-26 on COX-2 expression.

We next determined the effects of AC2-26 on MMDD1 COX-2 mRNA expression and tested whether or not CsH or WRW4 would block the effects of dexamethasone on COX-2 expression. AC2-26 reduced MMDD1 COX-2 mRNA abundance to a similar extent as Dex. The effect of Dex treatment was significantly ameliorated by a concomitant treatment with CsH, while CsH alone had no effect (Fig. 11). Co-application of Dex and WRW4 had no effect (data not shown).

DISCUSSION
Previous studies have documented the presence of the calcium-dependent enzymes PLA2G4, COX-2, and PGE2 synthase in macula densa cells. The regulated local generation of PGE2 by the macula densa plays a major role in the modulation of renal hemodynamics and renin production (6, 22, 23, 41, 42). Previous studies by McKanna et al have demonstrated ample expression of the endogenous anti-inflammatory protein ANXA1 in cells of the macula densa of rats (29). Since prostaglandin production in inflammatory cells is effectively inhibited by ANXA1 it seems reasonable to speculate that this protein may also participate in the regulation of prostaglandin synthesis in macula densa cells (11). The goal of the present experiments was to characterize the mechanisms governing ANXA1 expression and its effect on juxtaglomerular COX-2 and renin expression.

Our observations confirm that ANXA1 is heavily expressed in the macula densa. In addition, we show that, like COX-2 and NOS1, ANXA1 expression is increased in response to furosemide treatment, suggesting a role of macula densa salt transport for the regulation of ANXA1 expression. Subsequent colocalization studies revealed a high extent of ANXA1 and COX-2 coexpression that approached 100% in furosemide treated rats. Taken together, these results are in line with the proposed regulatory role of ANXA1 in macula densa prostaglandin synthesis. The regulation of ANXA1 expression is incompletely understood and may be cell-specific. In macula densa cells, the parallel regulation of ANXA1 and COX-2 and the high extent of coexpression suggest the presence of a common regulator for both the expression of ANXA1 and COX-2. Recent studies addressing the regulation of COX-2 expression in the macula densa have identified p38 MAP kinase as an activator of COX-2 expression (8, 46). The enzyme has also been shown to induce ANXA1 expression in a human colon carcinoma cell line.
Since p38 MAP kinase activity is induced in the macula densa of rats fed a low salt diet and in MMDD1 cells following exposure to a medium containing reduced chloride concentrations, p38 MAP kinase may be responsible for the parallel regulation of ANXA1 and COX-2 in the furosemide treated animals.

In addition to the concordant regulation of ANXA1 and COX-2 by similar intracellular pathways, evidence from inflammatory cells suggests that ANXA1 inhibits COX-2 expression as part of its strongly anti-inflammatory spectrum of actions. For example, the regulation of ANXA1 and COX-2 expression has been shown to be dissociated in inflammatory cells treated with dexamethasone (11). We replicated this finding in our studies on MMDD1 cells where dexamethasone treatment caused a reduction of COX-2 levels, but augmented the excretion and expression of ANXA1. The underlying mechanisms of these effects and the pathophysiological importance of dexamethasone-induced downregulation of macula densa COX-2 are still unclear.

Downregulation of COX-2 by ANXA1 in MMDD1 cells was confirmed by transiently transfecting cells with rat ANXA1. The transfection caused the expected increase in ANXA1 expression, and this was accompanied by a significant reduction of COX-2 mRNA levels. Together, these results suggest that macula densa ANXA1 expression may be regulated by multiple factors. Further studies are required to elucidate the nature of these factors and their contribution to the regulation of ANXA1.

The relationship between ANXA1 on COX-2 expression has also been extensively studied in ANXA1 knockout mice in which stimulation of COX-2 expression was found in some but not all tissues suggesting a cell-specific effect of ANXA1 on COX-2 expression (1, 13, 15). In cultured microglial cells the N-terminal ANXA1 peptide AC2-26 caused a significant downregulation of COX-2 expression and PGE2 synthesis.
In addition, the inhibitory effects of glucocorticoid treatment on COX-2 expression were shown to at least partially depend on ANXA1 (31). Thus, our results suggest that the macula densa belongs to the tissues in which ANXA1 exerts an inhibitory effect on COX-2 expression. The functional relevance of ANXA1 for juxtaglomerular signal transduction is furthermore highlighted by our finding of increased renin expression levels in ANXA1 knockout mice.

Previous studies have shown that the biological effects of ANXA1 depend on a regulated secretion of the protein and the subsequent activation of the FPR family of receptors (11). In the present studies we demonstrate that both dexamethasone treatment and ANXA1 overexpression in MMDD1 cells significantly increased ANXA1 secretion into the medium. The mechanism involved in the secretion of ANXA1 by MMDD1 cells has not been elucidated. In macrophages and endocrine cells, ANXA1 secretion required the action of ABCA1 and was effectively blocked by probenecid or glibenclamide (7, 44). Whereas we showed the presence of ABCA1 mRNA in both mouse kidney and MMDD1 cell extracts, we were not able to show an effect of glibenclamide or probenecid on ANXA1 externalization in response to dexamethasone in our experimental setting (data not shown). According to an alternative hypothesis, ANXA1 externalization is a result of cells shedding ANXA1-containing microparticles (2, 12). Interestingly, macula densa cells have been shown to rapidly change cell volume in response to changes in the salt content of the tubular fluid. High salt concentrations cause significant swelling and low salt concentration cause shrinkage in an isolated JGA preparation (21). We therefore speculate that these changes in membrane surface area may be associated with shedding of microparticles as has been shown for HEK 293 cells (2).
How extracellular ANXA1 could exert its effects has received widespread attention (11). Upon secretion, ANXA1 becomes a target for proteolytic cleavage of the 26 N-terminal amino acids (25). The resultant peptide, as well as the full length protein, activate the FPR receptor family (30). The identity of the ANXA1 receptor in the mouse has not been unequivocally elucidated. In our hands, PCR analysis of FPR expression demonstrated the presence of mRNA for Fpr1, Fpr2 and Fpr3 in mouse kidney and MMDD1 cells. fMLF and AC2-26, which act as agonists of both receptors, caused a significant downregulation of COX-2 mRNA in MMDD1 cells and thus mimicked the effects of glucocorticoids and ANXA1 overexpression. Co-application of dexamethasone and the Fpr1 blocker CsH ameliorated the inhibitory effect of the glucocorticoid on MMDD1 COX-2 expression. In contrast, the Fpr2/Fpr3 antagonist WRW4 had no effect. Our results thus suggest that the inhibitory effect of ANXA1 on macula densa COX-2 expression requires the externalization of the protein and the subsequent activation of Fpr1.

In summary, we have shown that the anti-inflammatory protein ANXA1 is expressed in the macula densa where it is coexpressed with NOS1 and COX-2. Treatment of cultured MMDD1 cells with dexamethasone causes an upregulation of ANXA1 expression and an augmented secretion of the protein. Extracellular ANXA1 may act via binding to formyl peptide receptors, presumably Fpr1, to inhibit macula densa COX-2 expression. Taken together these results suggest that ANXA1 serves as an important intrinsic regulator of macula densa prostaglandin synthesis.

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DISCLOSURES
The authors have no interest conflicts to report.

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**FIGURE LEGENDS**

**Fig. 1.** Expression of annexin A1 (ANXA1), cyclooxygenase 2 (COX-2) and phospholipase A2 group IVA (PLA2G4) in rat kidney and cultured mouse MMDD1 cells. A,B: Representative high power confocal micrographs documenting the intracellular localization of immunoreactive ANXA1 protein in rat macula densa (A) and cultured
mouse MMDD1 cells (B) with an even distribution throughout the cytoplasm and, less
pronounced, in the nucleus. C,D: Representative high power confocal micrographs
demonstrating cytosolic and perinuclear localization of immunoreactive COX-2 protein in
rat macula densa (C) and cultured mouse MMDD1 cells (D). E,F: Representative high
power confocal micrographs demonstrating cytosolic and perinuclear localization of
immunoreactive PLA2G4 protein in rat macula densa (E) and cultured mouse MMDD1
cells (F). G marks glomeruli and macula densa is marked by arrows between flanking
lines in A, C, and E. Cy3 immunofluorescence staining; original magnification x630.

Fig. 2. Expression profile of mouse kidney and MMDD1 cells. A: representative
micrograph of annexin A1 (ANXA1) in situ hybridization on control rat kidney showing
mRNA signal in podocytes, cells of the Bowman capsule and in the macula densa; ISH,
original magnification x400, glomerulum is marked with G. B: PCR analysis of murine
kidney (mouse) and MMDD1 cDNA samples demonstrating expression of ANXA1,
cyclooxygenase 2 (COX-2) and the ANXA1 receptors formyl peptide receptor 1 (Fpr1),
Fpr2 and Fpr3 in both sources. ATP binding cassette transporter 1 (ABCA1) which may
mediate ANXA1 secretion is expressed as well. Samples without cDNA were run in
parallel and served as negative controls (NK).

Fig. 3. Effect of furosemide (Furo) treatment on macula densa annexin A1 (ANXA1)
expression. A,B: high power micrographs of ANXA1 immunohistochemistry in control (A)
and Furo treated rats (B) revealed a significantly increased number of ANXA1
immunoreactive macula densa cells in the treated animals (between flanking lines in A
and B; immunohistochemistry; original magnification 400x). C. Quantification of ANXA1
expressing macula densa cells by cell counting with normalization to the total number of glomeruli confirmed increased number of immunoreactive cells. Data are means ± SEM from n = 4 rats/group; * p < 0.05.

**Fig. 4.** Double labeling immunofluorescence staining of annexin A1 (ANXA1) and nitric oxide synthase 1 (NOS1) in control and furosemide treated rats. Double labeling of ANXA1 (A,D) and NOS1 (B,E) in control (A-C) and furosemide treated (D-F) rats demonstrating frequent coexpression of the two products without significant, treatment-associated differences in the extent of coexpression. In the merged images (C,F) red signal indicates COX-2 and green signal ANXA1. Blue signals mark DAPI-stained nuclei. Double labeling immunofluorescence staining; original magnification x400.

**Fig. 5.** Double labeling immunofluorescence staining of annexin A1 (ANXA1) and cyclooxygenase 2 (COX-2) in control and furosemide treated rats. Double labeling of ANXA1 (A,D) and COX-2 (B,E) in control (A-C) and Furo treated (D-F) rats demonstrating frequent coexpression of the two products without significant, treatment-associated differences in the extent of coexpression. In the merged images (C,F) red signal indicates COX-2 and green signal ANXA1. Blue signals mark DAPI-stained nuclei. Double labeling immunofluorescence staining; original magnification x400.

**Fig. 6.** Expression of annexin A1 (ANXA1) and cyclooxygenase 2 (COX-2) in ANXA1 knockout mice. A, B: representative high power confocal micrographs demonstrating strong ANXA1 signal in the macula densa of control mice (between flanking lines in A) and a complete absence of immunoreactive product in the macula densa of ANXA1
knockout mice (between flanking lines in B). C, D: representative high power confocal micrographs documenting an increased number of COX-2 immunoreactive macula densa cells in ANXA1 knockout mice (D) as compared to controls (C). Cy3 immunofluorescence staining; original magnification x400 (A-D), G marks glomeruli in A-D. E: Quantification of COX-2 expressing macula densa cells by cell counting and normalization to the total number of glomeruli confirmed increased number of immunoreactive cells. Data are means ± SEM from n = 6 mice/group; * p < 0.05.

**Fig. 7.** Renin mRNA expression in ANXA1 knockout mice. A, B: representative high power micrographs documenting an increased abundance of renin mRNA in the juxtaglomerular region of ANXA1 knockout mice (B) as compared to controls (A). Non-radioactive mRNA in situ hybridization; original magnification x400. C: Quantification of renin mRNA signals and normalization to the total number of glomeruli confirmed increased number of immunoreactive cells. Data are means ± SEM from n = 5 mice/group; * p < 0.05.

**Fig. 8.** Effect of transient overexpression of annexin A1 (ANXA1) on MMDD1 cell cyclooxygenase 2 (COX-2) mRNA levels. A: quantification of ANXA1 mRNA in MMDD1 cells transfected with a plasmid coding for full-length ANXA1 under the control of a CMV promotor revealed a significant 8.4fold induction of ANXA1 mRNA levels. B: Concomitantly, COX-2 mRNA levels were reduced. C: Western blot analysis of ANXA1 content of cell culture supernatant of ANXA1 transfected cells and controls revealed an increased abundance of ANXA1 in the supernatant of the transfected cells. Taqman
realt time PCR analysis (A and B) and ANXA1 Western blot (C). All experiments were run
in triplicates and repeated at least three times. Data are means ± SEM; *P<0.05.

Fig. 9. Effect of the formyl peptide receptor agonist fMLF on MMDD1 cyclooxygenase 2
(COX-2) expression. A. Western blot analysis revealed reduced COX-2 protein
abundance following treatment with fMLF (A1; 8h, 0.1 µM). Beta actin (β-actin) content
of the cell homogenates was determined in parallel and served as loading control (A2).
B: Quantification of COX-2 mRNA by Taqman real time PCR revealed significantly
reduced levels in the treated cells. All experiments were run in triplicates and repeated
at least three times. Data are means ± SEM; *P<0.05.

Fig. 10. Effect of dexamethasone (Dex) treatment on MMDD1 cyclooxygenase 2 (COX-2)
and annexin A1 (ANXA1) expression and ANXA1 secretion. A. Western blot analysis
of ANXA1-content of MMDD1 cells (A2) and cell culture supernatant (A1) of control and
Dex treated cells (4h, 0.1 µM) revealed an increased abundance of ANXA1 in the
supernatant of the treated cells whereas total ANXA1 content of the cells remained
stable. Beta actin content of cell homogenates was determined in parallel and served as
loading control (A3). B: Longer Dex treatment (8h, 0.1µM) caused accumulation of
ANXA1 mRNA (B) and a decreased COX-2 mRNA abundance (C) as determined by
quantitative Taqman real time PCR. All experiments were run in triplicates and repeated
at least three times. Data are means ± SEM; *P<0.05.

Fig. 11. Effect of Cyclosporin H (CsH) on Dexamethasone (Dex) induced changes of
MMDD1 cyclooxygenase 2 (COX-2) expression. Quantitative Taqman real time PCR
demonstrated a significant downregulation of MMDD1 COX-2 mRNA following treatment with AC2-26 (8h, 50µM) or Dex (8h, 0.1µM). The effect of Dex was attenuated by concomitant treatment with the selective formyl peptide receptor 1 antagonist CsH (Dex+CsH; 8h, both 0.1µM). Treatment with CsH alone had no effect on MMDD1 COX-2 expression. All experiments were run in triplicates and repeated at least three times.

Data are means ± SEM; *P<0.05.
Figure 3

C  
ANXA1 protein abundance

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<tr>
<th></th>
<th>Control</th>
<th>Furo</th>
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<td>cells / 100 glomeruli</td>
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*Significant difference between groups
Figure 6

E

COX-2 protein abundance

WT  ANXA1 -/-

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*
Figure 7

Renin mRNA abundance

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<th>ANXA1 -/-</th>
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<td>positive JGA / 100 glomeruli</td>
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<td>30</td>
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A B

*
Figure 8

A  ANXA1 mRNA abundance

B  COX-2 mRNA abundance

C  ANXA1, MMDD1 supernatant

control  ANXA1

Transfection
Figure 9

A

COX-2, MMDD1 homogenates

A1

β-actin, MMDD1 homogenates

A2

control fMLF

B

COX-2 mRNA abundance

x-fold of control

control fMLF
**Figure 10**

A

ANXA1, MMDD1 supernatant

A1

ANXA1, MMDD1 homogenates

A2

β-actin, MMDD1 homogenates

A3

count

Dex

B

ANXA1 mRNA abundance

C

COX-2 mRNA abundance

x-fold of control

count

Dex

*
Figure 11

COX-2 mRNA abundance

control  AC2-26  Dex  CsH  Dex+CsH

* x-fold of control