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Synergistic Induction of Osteopontin by Aldosterone and Inflammatory Cytokines in Mesangial Cells

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Abstract Hypertensive nephrosclerosis is characterized by activation of the renin–angiotensin–aldosterone system in combination with an inflammatory response characterized by an infiltration of T-cells and mononuclear cells, which release proinflammatory cytokines like IL-1β/TNFα. In various models of experimental hypertensive disease the chemokine osteopontin (OPN) enhances further leukocyte infiltration. Therefore, we investigated the induction of OPN expression in renal mesangial cells (MCs) by aldosterone and the inflammatory cytokines IL-1β/TNFα. Incubation with aldosterone resulted in a time- and concentration-dependent increase in OPN mRNA and protein. OPN mRNA expression followed a biphasic time course with an early increase between 4 and 8 h and the second phase starting at 14 h. The early phase was independent of protein synthesis, indicating a direct effect of aldosterone. Aldosterone-mediated induction of OPN was prevented by spironolactone, indicative of a receptor-mediated aldosterone effect. The mineralocorticoid receptor (MR) was identified in MCs by RT-PCR and immunoprecipitation, and shown to interact with a putative aldosterone-response element of the OPN promoter. The proinflammatory cytokines IL-1β and TNFα only marginally affected OPN expression in MCs. However, coincubation of aldosterone and the cytokines synergistically increased OPN mRNA and protein levels. Since the synergistic effect on OPN mRNA was inhibited by diphenyleneiodonium, we assume an involvement of reactive oxygen species (ROS). We conclude that the chemokine OPN is a target gene of aldosterone in renal MCs, which is activated via the MR, and that proinflammatory cytokines enhance aldosterone-dependent OPN expression. In vivo, this may result in further leukocyte infiltration aggravating hypertensive nephrosclerosis. J. Cell. Biochem. 103: 615–623, 2008.

Key words: aldosterone; osteopontin; mesangial cells

The mineralocorticoid hormone aldosterone plays an essential role in the regulation of salt and water homeostasis by interaction with mineralocorticoid receptors (MR) expressed in renal tubular cells [Fuller and Young, 2005]. However, the MR is also expressed in other renal and extrarenal cells and tissues, extending the functional role of aldosterone beyond its mineralocorticoid action. Most importantly, aldosterone has been implicated in the development of vascular inflammation and myocardial fibrosis [Struthers, 2002]. Evidence for the pathophysiological role of aldosterone in left ventricular fibrosis was obtained from animal studies [Sen et al., 1977; Modena et al., 2001], but it was also shown in man that chronic elevation of plasma aldosterone contributes to heart failure [Cohn et al., 2003]. There is recent evidence that aldosterone effects are not restricted to the heart. The fact that mice transgenic for the human MR developed a nephropathy with decreased renal potassium excretion, indicates a crucial role of aldosterone during renal fibrosis [Le Menuet et al., 2001]. In a recent report, Lai et al. [2006] described aldosterone-mediated fibronectin synthesis in mesangial cells (MCs), indicative

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of a direct profibrotic effect of aldosterone. Inhibition of aldosterone receptors has been shown to ameliorate various kidney diseases in experimental models and also in man [Remuzzi et al., 2005].

During the onset of hypertensive nephrosclerosis an infiltration of mononuclear leukocytes and T-cells into glomeruli and renal interstitium is observed [Mai et al., 1993], regulated by an increased expression of adhesion molecules like ICAM-1 and LFA-1 [Mai et al., 1996], as well as by an induction of chemokines like MCP-1 or osteopontin (OPN) [Hilgers et al., 2000; Hartner et al., 2001; Gauer et al., 2003]. Aldosterone has been implicated in the pathogenesis of progressive renal diseases, independently from its effects on blood pressure [Hostetter et al., 2001]. Most of these studies are based on the beneficial effects of MR antagonists such as spironolactone, whereas the molecular targets mediating the adverse effects of aldosterone are less well defined in the kidney.

One of the potential molecular targets of aldosterone participating in inflammatory and fibrotic processes in the kidney is the matricellular protein OPN [Giachelli et al., 1998; Ophascharoensuk et al., 1999]. OPN is a multifunctional protein [Mazzali et al., 2002], involved in the regulation of bone turnover and urine stone formation, which also modulates inflammatory processes, for example, by attracting mononuclear cells [Giachelli et al., 1998; Weber et al., 2002] or by regulating T-cell immune response [Ashkar et al., 2000]. Aldosterone/salt-induced renal injury was related to macrophage infiltration, upregulation of OPN and increased circulating OPN levels [Blasi et al., 2003]. In an aldosterone-dependent animal model of hypertensive nephrosclerosis, we detected upregulation of OPN mRNA and protein in tubular and MCs [Hartner et al., 2001]. While OPN was expressed abundantly in cortical tubular epithelial cells, OPN RNA and protein synthesis was only observed in severely damaged glomeruli suggesting that multiple stimuli might be involved in the regulation of OPN expression in glomerular cells. Previously we observed increased OPN expression in rat MC in response to stimulation with platelet-derived growth factor (PDGF) or the glucocorticoid dexamethasone in vitro [Goppelt-Struebe et al., 2000], whereas aldosterone as direct inducer of OPN in MC has not yet been investigated. It was thus the aim of the present study to further analyze the molecular mechanisms involved in the regulation of OPN expression in MCs, which might underlie the induction of OPN synthesis in the course of hypertensive nephrosclerosis.

METHODS

Cell Culture

Rat MCs were isolated and cultured as described [Lovett et al., 1983]. MCs were cultured in DMEM supplemented with 10% FCS, 5 μg/ml bovine insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin. Experiments were performed with MCs at passages 5 through 20. Prior to stimulation, cells were cultured for 3 days in culture medium containing 0.5% FCS. Immortalized rat tubular cells (WKPT) were a kind gift of Dr. U. Hopfer, Case Western Reserve University, Cleveland, Ohio, and were cultured in DMEM/HamF12 as described earlier [Woost et al., 1996].

Northern Blot Analysis

Total RNA was isolated from MC cells using Trizol reagent (Invitrogen, Karlsruhe, Germany). Fifty micrograms of RNA was loaded for each lane on a 1% denaturing agarose gel (0.02 M MOPS pH 7.0 and 2.2 M formaldehyde). After electrophoresis RNA was transferred to a nylon membrane (Byodyne B, Gelman) by capillary blotting. After hybridization the membrane was exposed to Kodak X-Omat AR film. The signal was quantified using the ImageJ software (NIH) and normalized to the signal of glycerinaldehyde-3-phosphate dehydrogenase (GAPDH).

Probes

A 523 bp OPN cDNA and a 300 bp rat GAPDH cDNA probe were amplified by RT-PCR from total RNA of serum-stimulated rat MCs and cloned into a pCRScript vector (Stratagene, Heidelberg, Germany). ^{32}P-labeled cDNA probes for OPN and rat GAPDH were generated using an Amersham Megaprime kit (Amersham Pharmacia Biotech, Freiburg, Germany).

PCR

For detection of the MR, the following primers generated from the published sequence of the rat MR were used. Forward primer:

5′-TCCACACTGAGTGCTATGA, reverse primer: 5′-GTGACAGCTCCGCGATA. PCR was performed using an annealing temperature of 52°C and 30 cycles.

Western Blot Analysis

Cell culture medium was aspirated, the cell layer washed three times with PBS, and cells were scraped into 400 μl lysis buffer (10 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.1% Tween 20, 5 mM PMSF, 10 mM N-ethylmaleimide, and 1 mM benzamidine). Fifty micrograms of total cellular protein were loaded onto a 7.5% SDS-gel. After electrophoresis the proteins were transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% dry milk in Tris-buffered saline, 0.1% Triton X-100, 0.05% Tween 20 (TTBS) for 2 h and incubated with a mouse anti-rat OPN antibody (4.5 μg/ml) (MPIIIB10 obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA.) overnight at 4°C. The primary antibody was detected by a rabbit anti-mouse Ig antibody coupled to horseradish peroxidase (Amersham, Braunschweig) diluted 1:5,000 in TTBS and visualized using an ECL-Kit (Amersham, Braunschweig). OPN signals were quantified using the Quantiscan software (Biosoft, Cambridge, UK).

Electromobility Shift Assay

To prepare nuclear extracts MCs were washed with PBS and detached with 0.1 mM EDTA in PBS. After centrifugation (30 s, 10,000*g*) the cell pellet was dissolved in 300 μl ice cold hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF) and incubated on ice for 20 min. Ten microliters of a 10% NP-40 solution were added and the cells vortexed for 15 s. After a 2 min centrifugation step (10,000*g*) the pellet containing the nuclei was lysed in 70 μl ice cold extraction buffer for 20 min. The lysate was centrifuged for 5 min at 10,000*g*. Binding reactions were carried out in 10 mM Tris-HCl (pH 7.5), 70 mM KCl, 5 mM dithiothreitol, 0.2 mM EDTA, 4% glycerol, and 7 mg/ml salmon sperm DNA, using 5×10^4 cpm of the DNA probe and 10 μg of crude nuclear extracts in a total volume of 20 μl. The reaction mixtures were incubated for 30 min at room temperature and separated on a 4.8% polyacrylamide gel ($0.5 \times$ TBE) at 15 V/cm. An oligonucleotide consisting of a putative mineralocorticoid responsive element in the rat OPN promoter (5′-GGG GTT ACA AAG AGT CCT GGA A-3′) was used to assess MR binding. The double stranded probes of wild-type and mutated (5′-GGG GTT AGA TAG AGT CGT GGA A-3′) MR responsive element were created by annealing of synthetic forward and reverse oligonucleotides. The probe was end labeled using ^{32}P-ATP and T4 polynulceotide kinase (New England BioLabs). For competition a 20-fold excess of either unlabeled wild-type or mutated DNA was added.

Immunoprecipitation

Confluent 12 cm dishes of MCs were rinsed with ice cold PBS and scraped into 500 μl RIPA buffer (50 mM Tris/HCl, 150 mM NaCl, 2 mM PMSF, 2 mM N-ethylmaleimide, 10 mM benzamidine, and 10 mM EDTA). Four hundred micrograms cellular protein was diluted to 400 μl with RIPA and preabsorbed with 40 μl of sepharose 4CLB (Pharmacia). After centrifugation the cellular extracts were incubated for 2 h with 5 μg goat antibody specific for the rat MR (Santa Cruz: sc-6860) and precipitated with 30 μl protein-A sepharose (Pharmacia) for 2 h. After washing six times with RIPA, 30 μl $2 \times$ SDS sample buffer was added. All washing solutions contained protease inhibitors. Precipitates were analyzed on a 6% SDS gel and visualized by Western blot analysis using an anti-idiotypic mouse anti-rat MR antibody (ABR clone H10E4C9F) which recognizes the aldosterone binding site of the MR.

Statistical Analysis

The differences between the experimental groups were determined by Mann–Whitney *U*-tests or Kruskall–Wallis analysis of ranks followed by Dunn tests. *P*-values of less than 0.05 were considered to be statistically significant.

RESULTS

Long-Term Induction of OPN Gene Expression by Aldosterone

Incubation of serum-deprived MCs with aldosterone induced long-term upregulation of OPN gene expression. Increased steady state levels of OPN mRNA were detected upon incubation with different concentrations of aldosterone (10^{-7}–5×10^{-6} M) for 24 and 48 h

(Fig. 1). Dexamethasone (10^{-6} M), which has been shown earlier to induce OPN in MCs [Goppelt-Struebe et al., 2000], was less effective than aldosterone at these time points. Angiotensin II (10^{-7} M), which induced OPN in tubular cell lines [Ricardo et al., 2000], did not significantly increase the steady state levels of OPN mRNA in MCs (Fig. 1).

The accumulation of cellular OPN protein occurred with a major time lag compared to the induction of OPN mRNA. Even with high concentrations of aldosterone (10^{-6} M), only a small increase in OPN protein was detectable at 24 h (e.g., Fig. 6). At later time points, however, a prominent increase in cell-associated OPN was observed by Western blot analysis (Fig. 2). Compared to untreated cells, OPN levels were increased 10 ± 3.2-fold by 10^{-7} M aldosterone and 18 ± 5.1-fold by 10^{-6} M aldosterone. Similar but less pronounced effects were observed after 48 h (data not shown).

To further elucidate the aldosterone-mediated regulation of OPN synthesis, the initial phase of OPN gene expression was analyzed in more detail. Time course experiments revealed two distinct phases in the induction of OPN mRNA (Fig. 3). A first maximum was detectable at 8 h resulting in a threefold increase ($n = 7$). Between 8 and 14 h OPN mRNA levels decreased, followed by a secondary increase reaching an about eightfold induction ($n = 7$) after 24 h. Pretreating the cells with 10 μM cycloheximide to inhibit de novo protein synthesis did not affect aldosterone-mediated induction of OPN up to 14 h. The second phase, however, was almost completely prevented by cycloheximide, indicating involvement of additional aldosterone-induced proteins in the long-term increase of OPN mRNA expression. Reduction of OPN expression was not due to cytotoxic effects of cycloheximide as analyzed by trypan blue exclusion and an unchanged appearance of cell morphology (data not shown).

OPN is a Target Gene of Aldosterone

The early induction of OPN, which was independent of de novo protein synthesis, suggested direct genomic effects of aldosterone. Therefore, we further investigated interactions between the MR and the OPN promoter.

Expression of the MR in MC was confirmed by RT-PCR. Using total RNA from serum-deprived MCs as template, RT-PCR with rat MR specific primers revealed a product of the expected size and sequence. In contrast, no product was amplified when RNA of proximal tubular (WKPT) cells was used, which do not express the MR (Fig. 4A). The presence of MR protein

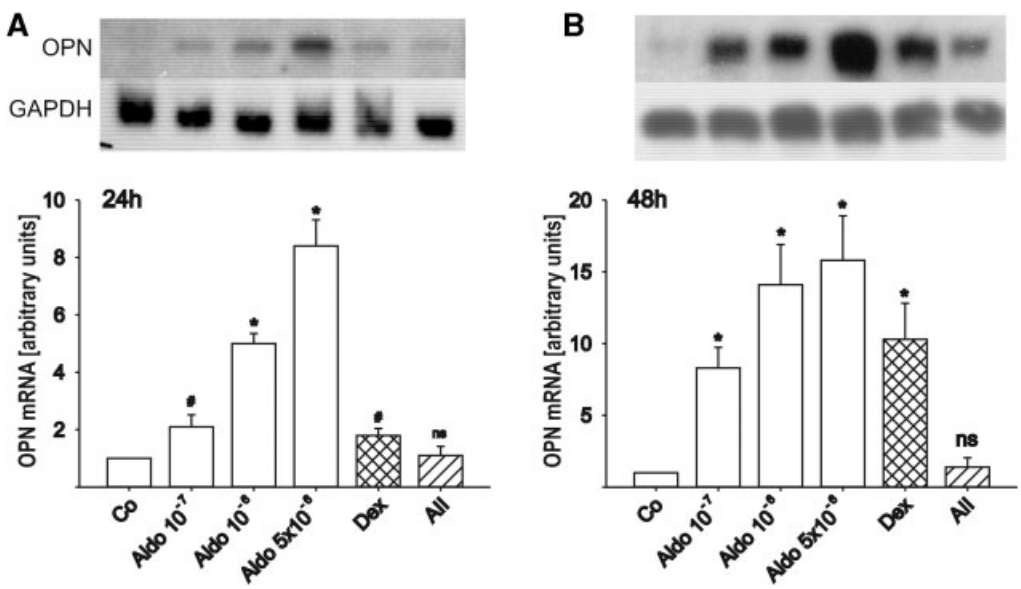

Fig. 1. Induction of OPN mRNA by aldosterone. **A**: Serum-deprived rat MCs were incubated for 24 and 48 h with aldosterone (10^{-7}, 10^{-6}, and 5×10^{-6} M) or with dexamethasone (10^{-6} M), or 10^{-7} M angiotensin II. GAPDH RNA is shown as control. **B**: Densitometric analysis of Northern blots from at least five independent experiments is shown. Data are expressed as the increase of steady state mRNA compared to the controls receiving ethanol as solvent control. Data represent mean ± SEM, $^{\#}P \leq 0.05$, $^{*}P \leq 0.01$, ns, non significant versus control.

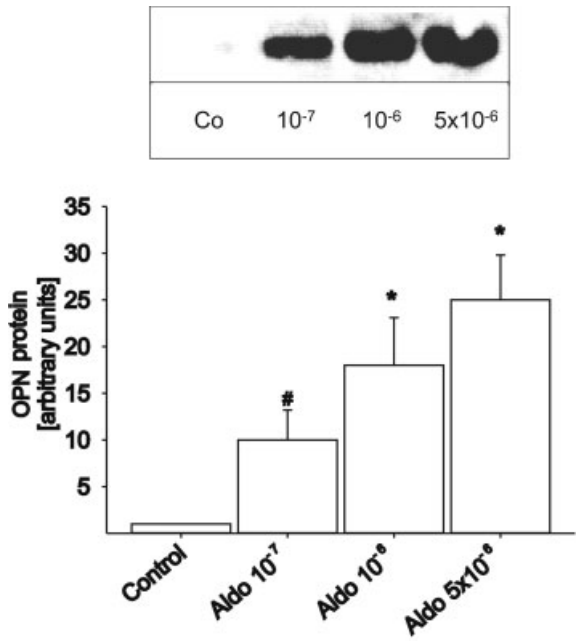

Fig. 2. Cellular OPN protein in serum-deprived rat MCs. Subconfluent cultures of rat MCs were treated with solvent (ethanol), or aldosterone at concentrations of 10^{-6}, 10^{-7}, and 5×10^{-6} M. After 5 days the protein of the MC layer was harvested. OPN was detected by Western blotting. Densitometric analysis of three independent experiments is shown. Data are expressed as increase in the OPN specific signal compared to the controls receiving solvent. $^{\#}P \leq 0.05$, $^{*}P \leq 0.01$.

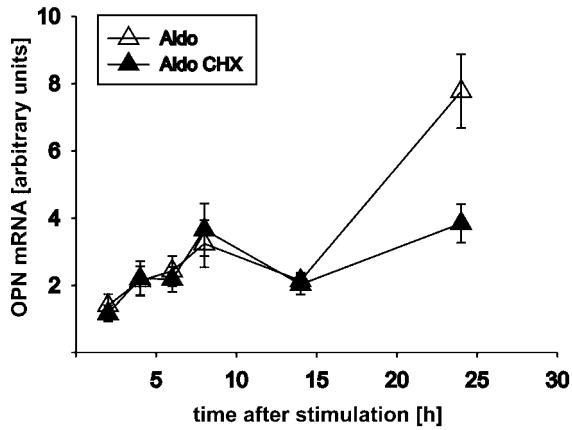

Fig. 3. Influence of cycloheximide on the aldosterone-dependent synthesis of OPN mRNA. MCs were pretreated for 30 min with 10^{-5} M cycloheximide (Aldo CHX) and then stimulated with 10^{-6} M aldosterone for the times indicated. Values are expressed as increase in OPN mRNA compared to controls (0 h aldosterone stimulation). Values represent the densitometric analysis of Northern blots of seven independent experiments. Data are expressed as mean ± SEM.

was shown by immunoprecipitation. MR was precipitated using a polyclonal antibody and was detected by a monoclonal antibody directed against the aldosterone binding site of the MR. The blot exhibited a single band of 110 kDa (Fig. 4B) corresponding to the known size of the MR.

The OPN promoter contains a putative steroid receptor binding site 1,984 bp upstream the OPN coding sequence. This sequence was used to investigate the direct interaction of MR and OPN promoter after aldosterone stimulation by electromobility shift assays. We detected a weak complex formation in the non-stimulated control cells (Fig. 4C). When the cells were incubated for 3 h with 10^{-7} M or 10^{-6} M aldosterone the signal strength of the complex was significantly increased. Due to similarity of the MR and the GR binding sites, complex formation was also detected when the cells were incubated with dexamethasone. In line with a common binding site of MR and GR, we observed additive OPN mRNA expression, when MCs were incubated with a combination of aldosterone and dexamethasone (data not shown). Competition with a 20-fold excess of the wild-type oligonucleotide totally inhibited the complex formation with nuclear extracts from MCs stimulated with 10^{-7} M aldosterone. Binding of the MR to the mutated oligonucleotide was barely stimulated by aldosterone (Fig. 4C), supporting the specificity of the complex.

Functionally, the binding of an active MR to the OPN promoter was supported by the inhibitory effect of the MR antagonist spironolactone. Spironolactone significantly inhibited the induction of OPN mRNA by aldosterone while it exhibited only a slight effect on the induction by dexamethasone, which did not reach statistical significance (Fig. 5).

Synergistic Induction of OPN by Aldosterone and Pro-Inflammatory Cytokines

Since aldosterone exerts profibrotic actions most probably in an inflammatory context, we investigated whether OPN induction by aldosterone is modulated by proinflammatory cytokines such as IL-1β and TNFα. While 10 U/ml IL1β/2 ng/ml TNFα by itself only slightly increased OPN mRNA (1.7 ± 0.2-fold) in serum-deprived MCs, costimulation with 10^{-6} M aldosterone and IL1β/TNFα resulted in a more than additive increase in OPN mRNA (Fig. 6A). Even more pronounced synergistic effects were observed at the protein level (Fig. 6B).

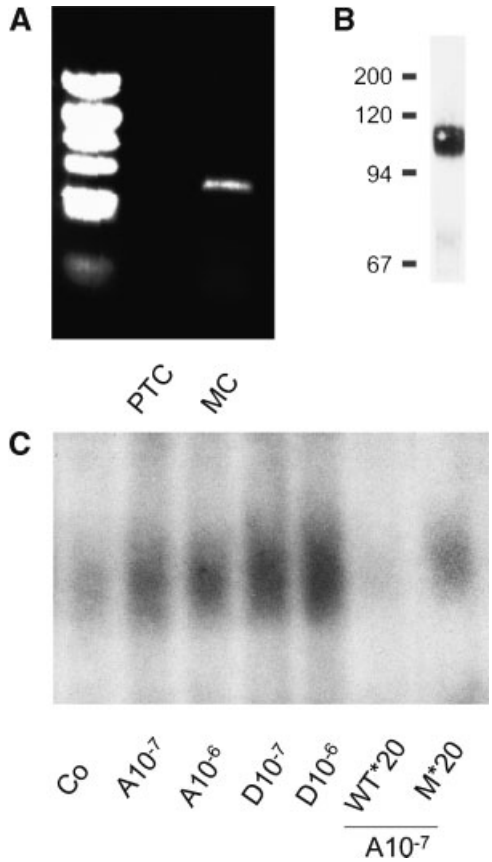

Fig. 4. Identification of the MR in rat MCs. **A**: RT-PCR was performed with RNA from rat MCs and from the immortalized rat proximal tubular cell (PTC) line WKPT using rat MR specific primers. After 30 cycles the PCR product was visualized by ethidium bromide staining. **B**: Immunoprecipitation of the MR using a rat specific goat anti-MR antibody. The precipitated protein was detected by Western blotting with an anti-idiotypic mouse antibody that binds to the rat MR. **C**: An electromobility shift assay was performed using a double stranded oligonucleotide derived from a putative steroid receptor binding site of the rat OPN promoter and nuclear extracts from MCs treated for 4 h with 0, 10^{-7}, and 10^{-6} M aldosterone (A) or dexamethasone (D). Unlabeled wildtype (WT*20) and non-binding mutated double-stranded oligonucleotides (M*20) were used in 20-fold excess to compete the binding of the radioactive oligonucleotide.

Incubation of the cells with IL1β/TNFα alone did not increase cellular OPN protein significantly (1.6 ± 0.9-fold increase). Coincubation of IL1β/TNFα with aldosterone (10^{-6} M) enhanced aldosterone-mediated induction from 7.6 ± 1.1-fold to 19.0 ± 0.9-fold compared to non-treated controls. This synergistic effect was even more pronounced at a lower aldosterone concentration of 10^{-7} M, which itself resulted only in a 2.2 ± 0.2-fold increase after 24 h, but together with IL1β/TNFα 10^{-7}M aldosterone led to a 17.1 ± 6.0-fold rise in OPN protein.

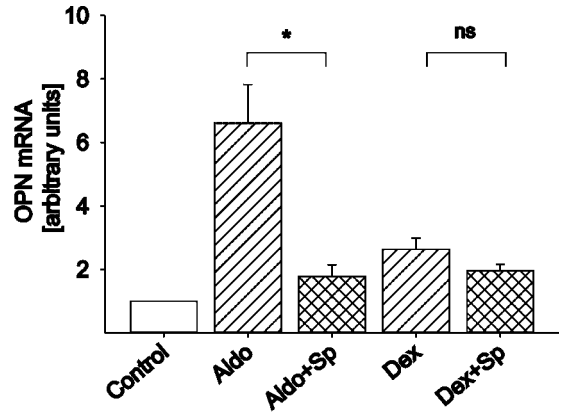

Fig. 5. Inhibition of the aldosterone-induced OPN expression by spironolactone. Serum-deprived MCs were treated for 24 h with 10^{-6} M aldosterone or dexamethasone, with or without 10^{-5} M spironolactone (Sp), which was added 30 min before steroid treatment. OPN mRNA was assessed by densitometric analysis of Northern blots of at least three independent experiments. Data represent mean ± SEM, $*P \leq 0.01$, ns, non significant.

IL1β/TNFα and aldosterone can induce reactive oxygen species (ROS). In order to determine, whether ROS signaling is involved in the synergistic effect of 10^{-6} M aldosterone and IL1β/TNFα, we incubated the cells for 24 h with 10 μM diphenyliodonium, an inhibitor of the NADPH oxidase (Fig. 7). Diphenyliodonium reversed the synergistic effect of IL1β/TNFα on the OPN mRNA induction, indicating a crucial role of ROS.

DISCUSSION

In vivo studies associated OPN expression with fibrosis and inflammation in the kidney. We could now show that OPN is a direct target gene of aldosterone in renal glomerular MCs. Furthermore, OPN was synergistically induced by aldosterone and pro-inflammatory cytokines providing a molecular basis for the upregulation of OPN in severe glomerular pathologies.

In accordance with its effects on salt and water balance in the kidney, aldosterone acts primarily on epithelial cells of the distal tubule and the collecting duct, and its receptor was assumed to be confined to these cells [Todd-Turla et al., 1993]. By binding assays, we had previously characterized high affinity binding sites for aldosterone in rat MCs [Schaefers and Goppelt-Struebe, 1996]. Recently expression of the MR in cultured MCs was shown and

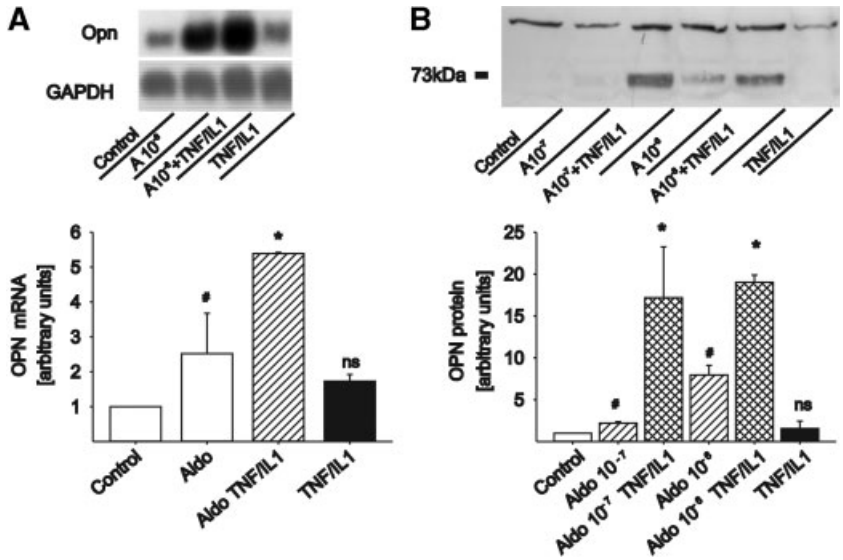

Fig. 6. Synergistic effects of aldosterone and TNFα/IL-1β on the expression of OPN mRNA and protein. **A**: Northern blot and densitometric analysis of MCs treated for 24 h with 10^{-6} M aldosterone, 10^{-6} M aldosterone plus 10 U/ml human recombinant IL1β/2 ng/ml human recombinant TNFα, IL1β/TNFα alone, or solvent (ethanol). Data from three independent experiments were expressed as mean ± SEM. **B**: MCs were stimulated with 10^{-7} M aldosterone, 10^{-6} M aldosterone, 10^{-7} M aldosterone plus IL1β/TNFα (10 U/ml/2 ng/ml), 10^{-6} M aldosterone plus IL1β/TNFα, IL1β/TNFα alone, or ethanol as control. One representative Western blot and the densitometric analysis of three independent blots are shown. Data are expressed as mean ± SEM. $^{\#}P \leq 0.05$, $^{*}P \leq 0.01$, ns, non significant.

confirmed in our study [Nishiyama et al., 2005; Terada et al., 2005; Lai et al., 2006].

We observed a two-phase time course of OPN RNA expression after aldosterone stimulation with a first peak after 8 h and a second more prominent induction starting at 14 h which was still ongoing after 24 h. The early phase was not influenced by inhibition of protein synthesis, indicating activation of non-genomic signaling pathways or direct transcriptional regulation of the OPN gene. Activation of the MAP kinase signaling pathway has been related to OPN induction [Chang et al., 2002; Xie et al., 2004]. In MCs activation of the MR led to a rapid activation of the MAP kinase pathway [Terada et al., 2005]. Activation of MAP kinases might thus contribute to the aldosterone-induced upregulation observed in this study. However, our kinetic studies did not support a mechanism based on signaling activation within minutes. Furthermore, using a putative MR response element located 1,984 bp upstream of the exon1 in the OPN promoter as probe, we could demonstrate a specific binding of the MR to OPN promoter DNA. These experiments establish OPN as a target gene of the MR in MCs.

The second phase of OPN induction was dependent on de novo protein synthesis indicative of additional target genes induced by aldosterone in MCs. The serum and glucocorticoid-regulated kinase (SGK) is an important target gene of aldosterone in renal epithelial cells [Kellner et al., 2003] mediating various long-term aldosterone effects. Since MCs can express SGK [Friedrich et al., 2002], it is a potential factor that may participate in the long-term regulation of OPN mRNA expression by aldosterone.

Recently it has become apparent that inhibition of aldosterone exhibits beneficial effects on kidney function and attenuates renal fibrosis [Norris and Vaughn, 2003] and has the potential to attenuate vascular inflammation [Han et al., 2006]. In accordance with these findings in vivo upregulation of OPN in glomeruli was observed in kidneys of hypertensive animals with signs of ongoing inflammation [Hartner et al., 2001]. In line with these findings we observed a prominent synergistic induction of OPN by a combination of aldosterone and the inflammatory cytokines IL-1β and TNFα. As mediators involved in the synergistic induction of OPN we identified ROS generated by NADPH oxidase. Inhibition of the enzyme by diphenyliodonium led to an almost complete disappearance of the synergistic OPN induction. Activation of ROS by

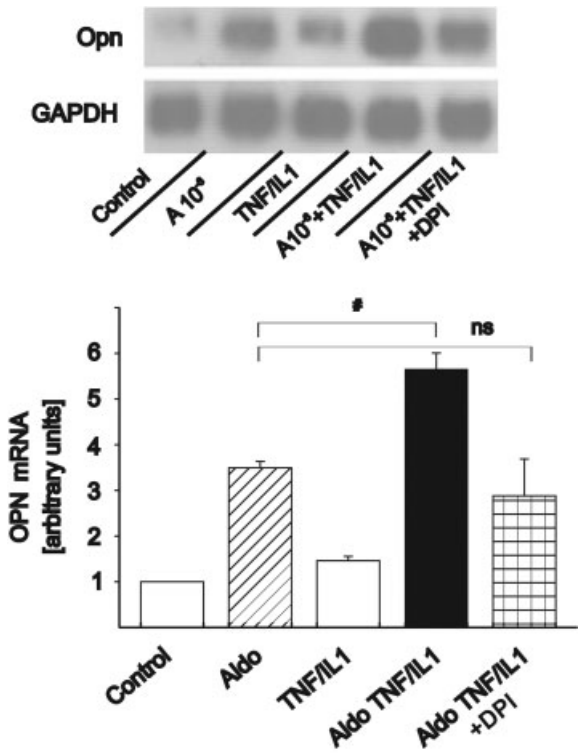

Fig. 7. Influence of reactive oxygen species on the synergistic induction of OPN mRNA expression by aldosterone and TNFα/IL-1β. Northern blot and densitometric analysis of MCs treated for 24 h with solvent (ethanol), 10^{-6} M aldosterone, 10^{-6} M aldosterone plus 10 U/ml human recombinant IL1β/2 ng/ml human recombinant TNFα, IL1β/TNFα alone, and 10^{-6}M aldosterone plus 10 U/ml human recombinant IL1β/2 ng/ml human recombinant TNFα plus 10 μM diphenyliodonium (DPI). Data from three independent experiments were expressed as mean ± SEM. $^{\#}P \leq 0.05$; ns, non significant.

inflammatory cytokines is well documented [Nian et al., 2004] and seems to be the essential for the upregulation of OPN by IL-1β/TNFα in our experiments. Furthermore, NADPH oxidase is activated in rat MCs by aldosterone in a MR-dependent manner [Miyata et al., 2005] and reactive oxygen generation activates downstream transcription factors including NFκB and AP-1 [Fiebeler and Luft, 2005], which have been shown to regulate OPN expression [Renault et al., 2005]. In addition to the direct MR effects at the OPN promoter, activation of NADPH oxidase dependent ROS signaling thus contributes to aldosterone-mediated OPN induction as shown by the partial reduction of aldosterone-mediated upregulation of OPN by diphenyliodonium.

The synergistic effects of aldosterone and IL-1β/TNFα may lead to an increased synthesis of OPN in vivo, which may be a part of a vicious cycle. The onset of hypertensive nephrosclerosis is characterized by an activation of the renin–angiotensin–aldosterone system leading to an infiltration of mononuclear leukocytes and T-cells into glomeruli and renal interstitium, which are able to induce proinflammatory cytokines. Together with aldosterone these cytokines potentially induce OPN. As a consequence of the chemotactic action of OPN more leukocytes are attracted, which release profibrotic factors. This mechanism may contribute to the detrimental effects of aldosterone observed in various kidney diseases.

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REFERENCES

Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, Rittling SR, Denhardt DT, Glimcher MJ, Cantor H. 2000. Eta-1 (osteopontin): An early component of type-1 (cell-mediated) immunity. Science 287:860–864.

Blasi ER, Rocha R, Rudolph AE, Blomme EA, Polly ML, McMahon EG. 2003. Aldosterone/salt induces renal inflammation and fibrosis in hypertensive rats. Kidney Int 63:1791–1800.

Chang PL, Tucker MA, Hicks PH, Prince CW. 2002. Novel protein kinase C isoforms and mitogen-activated kinase mediate phorbol ester-induced osteopontin expression. Int J Biochem Cell Biol 34:1142–1151.

Cohn JN, Anand IS, Latini R, Masson S, Chiang YT, Glazer R. 2003. Sustained reduction of aldosterone in response to the angiotensin receptor blocker valsartan in patients with chronic heart failure: Results from the Valsartan Heart Failure Trial. Circulation 108:1306–1309.

Fiebeler A, Luft FC. 2005. The mineralocorticoid receptor and oxidative stress. Heart Fail Rev 10:47–52.

Friedrich B, Warntges S, Klingel K, Sauter M, Kandolf R, Risler T, Muller GA, Witzgall R, Kriz W, Grone HJ, Lang F. 2002. Upregulation of the human serum and glucocorticoid-dependent kinase 1 in glomerulonephritis. Kidney Blood Press Res 25:303–307.

Fuller PJ, Young MJ. 2005. Mechanisms of mineralocorticoid action. Hypertension 46:1227–1235.

Gauer S, Hartner A, Hauser IA, Fierlbeck W, Eberhardt W, Geiger H. 2003. Differential regulation of osteopontin expression in the clipped and nonclipped kidney of two-kidney, one-clip hypertensive rats. Am J Hypertens 16: 214–222.

Giachelli CM, Lombardi D, Johnson RJ, Murry CE, Almeida M. 1998. Evidence for a role of osteopontin in

macrophage infiltration in response to pathological stimuli in vivo. Am J Pathol 152:353–358.
Goppelt-Struebe M, Wiedemann T, Heusinger-Ribeiro J, Vucadinovic M, Rehm M, Prols F. 2000. Cox-2 and osteopontin in cocultured platelets and mesangial cells: Role of glucocorticoids. Kidney Int 57:2229–2238.
Han SY, Kim CH, Kim HS, Jee YH, Song HK, Lee MH, Han KH, Kim HK, Kang YS, Han JY, Kim YS, Cha DR. 2006. Spironolactone prevents diabetic nephropathy through an anti-inflammatory mechanism in type 2 diabetic rats. J Am Soc Nephrol 17:1362–1372.
Hartner A, Porst M, Gauer S, Prols F, Veelken R, Hilgers KF. 2001. Glomerular osteopontin expression and macrophage infiltration in glomerulosclerosis of DOCA-salt rats. Am J Kidney Dis 38:153–164.
Hilgers KF, Hartner A, Porst M, Mai M, Wittmann M, Hugo C, Ganten D, Geiger H, Veelken R, Mann JF. 2000. Monocyte chemoattractant protein-1 and macrophage infiltration in hypertensive kidney injury. Kidney Int 58:2408–2419.
Hostetter TH, Rosenberg ME, Ibrahim HN, Juknevicius I. 2001. Aldosterone in progressive renal disease. Semin Nephrol 21:573–579.
Kellner M, Peiter A, Hafner M, Feuring M, Christ M, Wehling M, Falkenstein E, Losel R. 2003. Early aldosterone upregulated genes: New pathways for renal disease? Kidney Int 64:1199–1207.
Lai L, Chen J, Hao CM, Lin S, Gu Y. 2006. Aldosterone promotes fibronectin production through a Smad2-dependent TGF-beta1 pathway in mesangial cells. Biochem Biophys Res Commun 348:70–75.
Le Menuet D, Isnard R, Bichara M, Viengchareun S, Muffat-Joly M, Walker F, Zennaro MC, Lombes M. 2001. Alteration of cardiac and renal functions in transgenic mice overexpressing human mineralocorticoid receptor. J Biol Chem 276:38911–38920.
Lovett DH, Ryan JL, Sterzel RB. 1983. A thymocyte-activating factor derived from glomerular mesangial cells. J Immunol 130:1796–1801.
Mai M, Geiger H, Hilgers KF, Veelken R, Mann JF, Dammrich J, Luft FC. 1993. Early interstitial changes in hypertension-induced renal injury. Hypertension 22:754–765.
Mai M, Hilgers KF, Geiger H. 1996. Experimental studies on the role of intercellular adhesion molecule-1 and lymphocyte function-associated antigen-1 in hypertensive nephrosclerosis. Hypertension 28:973–979.
Mazzali M, Kipari T, Ophascharoensuk V, Wesson JA, Johnson R, Hughes J. 2002. Osteopontin—A molecule for all seasons. QJM 95:3–13.
Miyata K, Rahman M, Shokoji T, Nagai Y, Zhang GX, Sun GP, Kimura S, Yukimura T, Kiyomoto H, Kohno M, Abe Y, Nishiyama A. 2005. Aldosterone stimulates reactive oxygen species production through activation of NADPH oxidase in rat mesangial cells. J Am Soc Nephrol 16: 2906–2912.
Modena MG, Aveta P, Menozzi A, Rossi R. 2001. Aldosterone inhibition limits collagen synthesis and progressive left ventricular enlargement after anterior myocardial infarction. Am Heart J 141:41–46.
Nian M, Lee P, Khaper N, Liu P. 2004. Inflammatory cytokines and postmyocardial infarction remodeling. Circ Res 94:1543–1553.
Nishiyama A, Yao L, Fan Y, Kyaw M, Kataoka N, Hashimoto K, Nagai Y, Nakamura E, Yoshizumi M, Shokoji T, Kimura S, Kiyomoto H, Tsujioka K, Kohno M, Tamaki T, Kajiya F, Abe Y. 2005. Involvement of aldosterone and mineralocorticoid receptors in rat mesangial cell proliferation and deformability. Hypertension 45:710–716.
Norris K, Vaughn C. 2003. The role of renin-angiotensin-aldosterone system inhibition in chronic kidney disease. Expert Rev Cardiovasc Ther 1:51–63.
Ophascharoensuk V, Giachelli CM, Gordon K, Hughes J, Pichler R, Brown P, Liaw L, Schmidt R, Shankland SJ, Alpers CE, Couser WG, Johnson RJ. 1999. Obstructive uropathy in the mouse: Role of osteopontin in interstitial fibrosis and apoptosis. Kidney Int 56:571–580.
Remuzzi G, Perico N, Macia M, Ruggenenti P. 2005. The role of renin-angiotensin-aldosterone system in the progression of chronic kidney disease. Kidney Int Suppl 99: S57–S65.
Renault MA, Jalvy S, Potier M, Belloc I, Genot E, Dekker LV, Desgranges C, Gadeau AP. 2005. UTP induces osteopontin expression through a coordinate action of NFkappaB, activator protein-1, and upstream stimulatory factor in arterial smooth muscle cells. J Biol Chem 280:2708–2713.
Ricardo SD, Franzoni DF, Roesener CD, Crisman JM, Diamond JR. 2000. Angiotensinogen and AT(1) antisense inhibition of osteopontin translation in rat proximal tubular cells. Am J Physiol Renal Physiol 278:F708–F716.
Schaefers HJ, Goppelt-Struebe M. 1996. Interference of corticosteroids with prostaglandin E2 synthesis at the level of cyclooxygenase-2 mRNA expression in kidney cells. Biochem Pharmacol 52:1415–1421.
Sen S, Tarazi RC, Bumpus FM. 1977. Cardiac hypertrophy and antihypertensive therapy. Cardiovasc Res 11:427–433.
Struthers AD. 2002. Impact of aldosterone on vascular pathophysiology. Congest Heart Fail 8:18–22.
Terada Y, Kobayashi T, Kuwana H, Tanaka H, Inoshita S, Kuwahara M, Sasaki S. 2005. Aldosterone stimulates proliferation of mesangial cells by activating mitogen-activated protein kinase 1/2,cyclin D1, and cyclin A. J Am Soc Nephrol 16:2296–2305.
Todd-Turla KM, Schnermann J, Fejes-Toth G, Naray-Fejes-Toth A, Smart A, Killen PD, Briggs JP. 1993. Distribution of mineralocorticoid and glucocorticoid receptor mRNA along the nephron. Am J Physiol 264: F781–F791.
Weber GF, Zawaideh S, Hikita S, Kumar VA, Cantor H, Ashkar S. 2002. Phosphorylation-dependent interaction of osteopontin with its receptors regulates macrophage migration and activation. J Leukoc Biol 72:752–761.
Woost PG, Orosz DE, Jin W, Frisa PS, Jacobberger JW, Douglas JG, Hopfer U. 1996. Immortalization and characterization of proximal tubule cells derived from kidneys of spontaneously hypertensive and normotensive rats. Kidney Int 50:125–134.
Xie Z, Singh M, Singh K. 2004. ERK1/2 and JNKs, but not p38 kinase, are involved in reactive oxygen species-mediated induction of osteopontin gene expression by angiotensin II and interleukin-1beta in adult rat cardiac fibroblasts. J Cell Physiol 198:399–407.