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ORIGINAL ARTICLE

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Angiotensin II induces p27^{Kip1} expression in renal tubules in vivo: role of reactive oxygen species

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Abstract Previous studies have demonstrated that angiotensin II (ANG II) mediates cell cycle arrest of cultured renal tubular cells by induction of p27^{Kip1}, an inhibitor of cyclin-dependent kinases. However, it is not known whether ANG II exerts similar effects in vivo. Infusion of ANG II into naive rats for 7 days increased formation of reactive oxygen species in tubular cells of the kidney. Furthermore, ANG II infusion stimulated protein expression of p27^{Kip1} as detected by western blotting of tubular lysates and immunohistochemistry. Infusion of ANG II reduced tubular proliferation as detected by proliferating-cell nuclear antigen (PCNA) immunohistochemistry. The increase in p27^{Kip1} expression was not due to an increase in mRNA. Immunoprecipitation experiments revealed that the increased p27^{Kip1} protein associates with cyclin-dependent kinase 2. Coadministration of the radical scavenger dimethylthiourea abolished this ANG II mediated p27^{Kip1} expression without reducing systemic blood pressure. Furthermore, dimethylthiourea infusion attenuates the ANG II mediated G_1-phase arrest of tubular cells. However, infusion of norepinephrine did not induce reactive oxygen species or p27^{Kip1} expression, despite a significant increase in blood pressure. Thus ANG II induces p27^{Kip1} expression in renal tubular cells in vivo. This effect is mediated by reactive oxygen species. Since tubular hypertrophy depends on G_1-phase arrest and may promote subsequent development of interstitial fibrosis, administering oxygen radical scavenger may be a therapeutic tool to counteract ANG II dependent remodeling of renal tubular cells.

Keywords Angiotensin II · p27^{Kip1} · Tubular hypertrophy · Cell cycle arrest · Reactive oxygen species

Abbreviations *ANG* II: Angiotensin II · *CDK*: Cyclin-dependent kinase · *DMTU*: Dimethylthiourea · *MAP*: Mitogen-activated protein · *PCNA*: Proliferating-cell nuclear antigen · *SDS*: Sodium dodecyl sulfate

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Introduction

Compensatory hypertrophy of tubular cells occurs during chronic renal disease [1, 2]. This adaptive growth of surviving renal cells has been viewed as increasing single nephron performance to compensate for the loss of functional renal tissue [2]. However, there is increasing evidence that this hypertrophy is detrimental in the long-term run for renal function because initial tubular hypertrophy has been linked to the subsequent development of

interstitial fibrosis [2]. The mechanism of this tubular hypertrophy is still incompletely understood. We [3, 4], and others [5, 6] have previously demonstrated that the vasoactive factor angiotensin II (ANG II) stimulates cellular hypertrophy of cultured proximal tubular cells from varies species. This hypertrophy is caused by an arrest of tubular cells in the G_1 phase of the cell cycle [1, 3]. We also discovered that this cell cycle arrest is caused by ANG II induced protein expression of $p27^{Kip1}$ and inhibitor of cyclin/cyclin-dependent kinase (CDK) complexes [7]. $p27^{Kip1}$ binds to G_1-phase cyclin/CDK complexes and inhibits their kinase activity [7]. An ANG II mediated increase in reactive oxygen species such as superoxide anion (O^{-}_{2}), produced by an increase in membrane-bound NAD(P)H-oxidase, is partly responsible for the stimulated $p27^{Kip1}$ protein expression [8, 9]. However, these observations were derived solely from cell culture studies, and it remains unclear whether similar mechanism are operative in vivo. Therefore the present study was performed to investigate whether systemic ANG II infusion into naive rats stimulates $p27^{Kip1}$ expression. The results reveal that ANG II infusion for 7 days induced formation of O^{-}_{2} in isolated tubules. Furthermore, $p27^{Kip1}$ expression, as determined by western blotting from tubular lysates or by immunohistochemistry, was significantly stimulated by ANG II infusion. This $p27^{Kip1}$ induction was attenuated by concomitant infusion of the antioxidant dimethylthiourea (DMTU). These data suggest that ANG II modifies expression of CDK inhibitors in renal tubular cells through oxygen stress.

Materials and methods

Animal experiments

ANG II (Sigma, Deisenhofen, Germany), redissolved in sterile phosphate-buffered solution (PBS), was infused intraperitoneally at a rate of 250 ng/min into male Wistar rats (body weight 200 g) using osmotic minipumps (Alzet 2002, infusion rate 0.5 µl/h, Palo Alto, Calif., USA). In addition, some animals received a second minipump intraperitoneally with the antioxidant DMTU (Sigma; infusion rate 400 µg/h). Control animals were infused only with PBS. Additional rats were infused with norepinephrine (Sigma) at a rate of 600 ng/min. Systolic blood pressure was measured on awake, slightly restrained animals on day 6 using tail plethysmography. Rats were trained for blood pressure measurements. All animal experiments were approved by the local government, and experiments were performed in accordance with the animal guideline of the National Institute of Health.

Isolation of tubules

On day 7 of ANG II infusion animals were slightly anesthetized with ether, and the kidneys were perfused in situ via the aorta first with 20 ml ice-cold PBS, followed by perfusion with 20 ml ice-cold collagenase II (253 U/mg, Biochrome, Berlin, Germany) redissolved at 1 mg/ml in RPMI medium (Gibco-BRL, Eggenstein, Germany). Tubules were isolated by differential centrifugation using a Percoll (Pharmacia, Freiburg, Germany) gradient exactly as previously described [10]. The two bands near the cushion interface in the 45% iso-osmotic Percoll (density 1.020–1.025 g/ml) that contained long proximal tubules were removed and pooled [10]. All preparations were performed on ice. The final tubular preparation had a purity of greater than 95% as judged by light microscopy.

Measurement of $O_2$2 generation in intact tubules

Measurement of O^{-}_{2} generation in intact cells was performed as described previously using the lucigenin method [8]. Tubular suspension were washed in a modified Krebs buffer containing 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1 mM K_2HPO_4, 20 mM HEPES (pH 7.4), adjusted to protein content as measured by a modification of the Lowry method, and centrifuged. After washing, cells were resuspended in Krebs buffer containing 10 mM glucose and 1 mg/ml bovine serum albumin. Measurement of O^{-}_{2} generation was performed in a luminometer (LB 9501 from Berthold, Wildbad, Germany) and started by addition of 100 µl lucigenin (Sigma; final concentration 500 µM). Photon emission was counted every 15 s for up to 15 min. A buffer blank (reaction buffer with lucigenin, but without cells) was subtracted from each reading. To calculate the amount of O^{-}_{2} synthesized by intact cells a standard curve was generated as previously described [8], and total photon emissions at 10 min were converted into nM O^{-}_{2}.

Western blot analysis

A fraction of the tubular suspension was lysed in 100 µl lysis buffer [2% sodium dodecyl sulfate (SDS), 60 mM Tris-HCL, pH 6.8], and the lysate was cleared by centrifugation. Of the protein 50 µg (measured by a modification of the Lowry method) were denatured by addition of 5% glycerol, 5% mercapthoethanol, and 0.03% bromophenol blue and boiling for 10 min. After centrifugation the supernatants were loaded on a 12% SDS polyacrylamide gel. Low molecular weight marker (Rainbow marker, Amersham-Buchler, Braunschweig, Germany) served as molecular weight standard. After electrophoresis proteins were electroblotted semi-dry for 1 h at 0.8 mA/cm² to a polyvinylidene fluoride membrane (Hybond P, Amersham). Selected membranes were stained with Ponceau S to ensure equal loading and transfer of proteins. The blots were blocked in 5% nonfat dry milk in PBS containing 0.1% Tween 20 for 1 h at room temperature. A mouse monoclonal anti-$p27^{Kip1}$ antibody (Transduction Laboratories, Lexington, Mass., USA) was used in a 1:1000 dilution [8]. Incubation was carried out for 1 h at room temperature. The blots were then washed once for 15 min and twice for 5 min in PBS containing 0.1% Tween 20. After incubation with an anti-mouse secondary antibody coupled to horseradish peroxidase, blots were washed again for 15 min, and the detection was carried out using the ECL system (Amersham). To control for equal protein loading and transfer, blots were reincubated with an antibody against β-actin (Sigma, 1:2000 dilution), Exposed films were scanned with Fluor-S multi-imager (Bio-Rad Laboratories, Hercules, Calif., USA), and data were analyzed with the computer program Multi-Analyst from Bio-Rad [8]. Western blots were repeated four times with qualitatively similar result.

Immunoprecipitation experiments

Isolated tubules (final concentration 400 µg protein) were lysed in 200 µl cold immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCL, pH 7.4; 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonylfluoride, 0.5% NP-40). After centrifugation the supernatant was transferred to a new tube, and 5 µg of either monoclonal anti-CDK2 or anti-CDK4 (both Transdution Laboratories), 300 µl distilled water, and 500 µl immunoprecipitation buffer were added. After incubation in a shaking ice-bath for 1 h, 5 µg of a polyclonal rabbit anti-mouse antibody (Transduction Laboratories) was added to facilitate formation of immune complexes. After another 30 min on ice, 50 µl *Staphylococcus aureus* Cowan strain (Calbio-

chem, Bad Soden, Germany) was added, and tubes were incubated with agitation for 1 h [7]. Precipitates were then washed three times with immunoprecipitation buffer, and recovered pellets were suspended in 30 µl electrophoresis buffer (2% SDS, 60 mM Tris-HCl, pH 6.8; 100 mM dithiothreitol, 5% glycerol, 0.03% bromophenol blue), boiled for 5 min, and supernatants were finally loaded onto a 12% SDS polyacrylamide gel. Western blotting and detection of $p27^{Kip1}$ were performed as described above. Immunoprecipitation experiments were independently performed twice with qualitatively similar results.

Isolation of RNA and cDNA amplification

To detect minor changes in $p27^{Kip1}$ mRNA expression semiquantitative cDNA amplification after reverse transcription of total RNA was performed exactly as previously described using the housekeeping RNA of GAPDH as an internal control [7]. Isolated tubules from four kidneys of either control or ANG II infused rats were pooled and directly lysed in 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. Isolation of total RNA and reverse transcription were performed as previously described. For cDNA amplification the following primers were used: $p27^{Kip1}$ (5′GTCTAACGGGAGCCCGAGCCTGG3′; 5′GAAGGCCGGGCTTCTTGGGCG3′, size of the amplification product 560 bp), GAPDH (5′AATGCATCCTGCACCACCAA3′; 5′GTAGCCATATTCATTGTCATA3′, size of the amplification product: 512 bp). A total of 150 ng of the primers was used. Complete amplification mixes without the primers were equally distributed to separate tubes containing either $p27^{Kip1}$ or GAPDH primers. Reactions were performed with the GeneAmp kit (Perkin Elmer Cetus, Überlingen, Germany). Amplifications were conducted for 35 cycles with an annealing temperature of 60°C for 1.5 min, an extension step at 72°C for 1.5 min, and a denaturation step at 92°C. Of the reaction product 10 µl was run on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide. Gels were photographed and scanned with the Fluor-S multi-imager. The ratio between $p27^{Kip1}$ and GAPDH was calculated, and controls were assigned an arbitrary value of one. Reverse transcription and cDNA amplification were performed independently three times.

Immunohistochemistry

Paraffin sections 2 µm thick were prepared. For immunohistochemistry demonstration of $p27^{Kip1}$ sections were dewaxed, rehydrated, and microwave pretreated. Tissue sections were incubated with a 1:20 dilution of the mouse monoclonal anti-$p27^{Kip1}$ antibody. Staining for anti-PCNA (Dakopatts, M879, Hamburg, Germany) was used to assess proliferation. As additional controls, slides were incubated with normal mouse serum. The alkaline phosphatase–antialkaline phosphatase complex was used to visualize the primary antibody. Positive cells ($p27^{Kip1}$, PCNA) were evaluated by an investigator blinded to the experimental protocol. All positive nuclei from tubular cells were counted per 40 microscopy field of the renal cortex.

Statistical analysis

All values are presented as means ±SEM. Statistical significance among multiple groups was tested with nonparametric Kruskal-Wallis test. Individual groups were then tested using the Wilcoxon/Mann-Whitney test. A P value less than 0.05 was considered significant.

Results

Blood pressures and oxygen radical production

Infusion of ANG II for 7 days with osmotic minipumps significantly increased systolic blood pressure (Table 1).

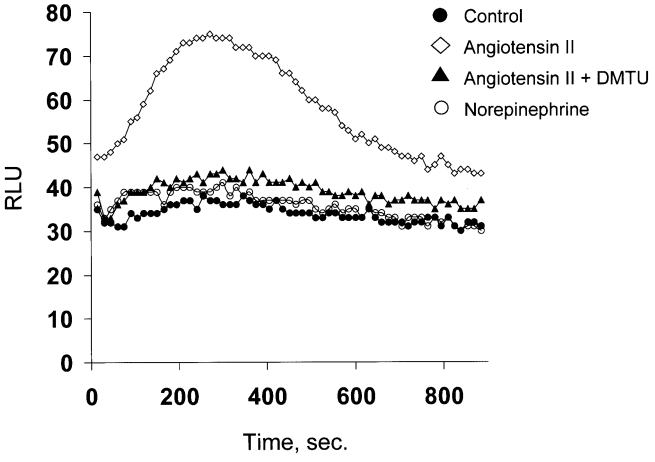

Fig. 1 Example of representative tracing of O^{-}_{2} measurement using the lucigenin method in intact tubules. Emission of relative light units (RLU) was corrected for unspecific luminescence in the absence of tubules and equal protein amounts of tubules were used. Measurement of O^{-}_{2} generation was started in the luminometer after addition of lucigenin, and measurements were performed every 15 s for up to 15 min. Tubules obtained from ANG II infused animals produced significantly more O^{-}_{2} than rats infused with PBS only. Coinfusion of DMTU abolished this stimulation. Intraperitoneal infusion of norepinephrine failed to stimulate tubular O^{-}_{2} generation. This tracing is representative for five experiments with qualitatively similar changes

Table 1 Summary of quantitative data (n=4–7; *n.m.* not measured)

	Controls	ANG II	DMTU	ANG II + DMTU	Norepinphrine
Systolic blood pressure (mmHg)	126±5	170±8*	120±8	186±16*	173±10*
O^{-}_{2} generation (nM min^{-1} μg^{-1} protein)	19.1±2.5	39.3±5.1*	n.m.	25.3±0.6**	23.0±0.6
Western blots desitometry (relative $p27^{Kip1}$ protein expression normalized to β-actin)	1.0±0.0	4.5±1.2*	n.m.	0.9±0.3**	1.2±0.6
Immunohistochemistry (positive $p27^{Kip1}$ nuclei per 40 fields)	35±2	57±3*	32±2	38±3**	31±3
Immunohistochemistry (positive PCNA nuclei per 40 fields)	0.9±0.2	0.5±0.1*	1.8±0.3*	1.2±0.2**	1.0±0.2

*P<0.05 versus controls, **P<0.05 versus ANG II only

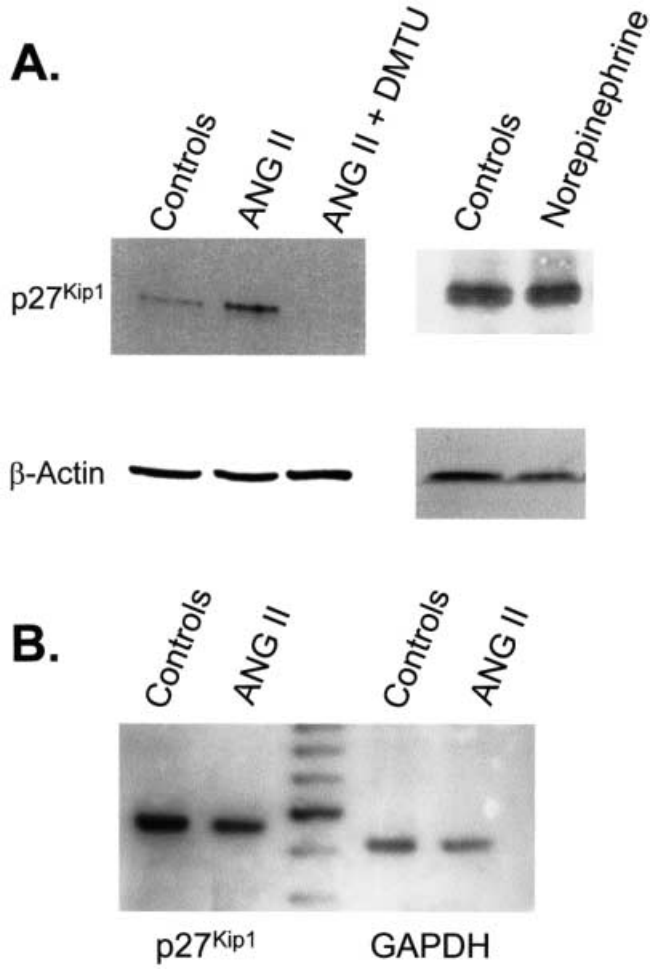

Fig. 2A,B Expression of $p27^{Kip1}$ in isolated tubules. **A** Western blot of tubular lysates incubated with an antibody against $p27^{Kip1}$. The blot was washed and reincubated with an antibody against β-actin to account for small variations in protein loading and transfer. Tubules isolated from ANG II infused animals expressed considerably more $p27^{Kip1}$ protein than those obtained from controls. Coinfusion of DMTU abolished this increase. Infusion of norepinephrine does not induce $p27^{Kip1}$. This blot is representative of four experiments with qualitatively similar results. **B** Semiquantitative cDNA amplification of reverse transcribed mRNA. Amplification of the housekeeping gene GAPDH served as control. As seen here, there was no increase in $p27^{Kip1}$ mRNA expression in tubules isolated from ANG II infused rats. This experiment was performed independently three times

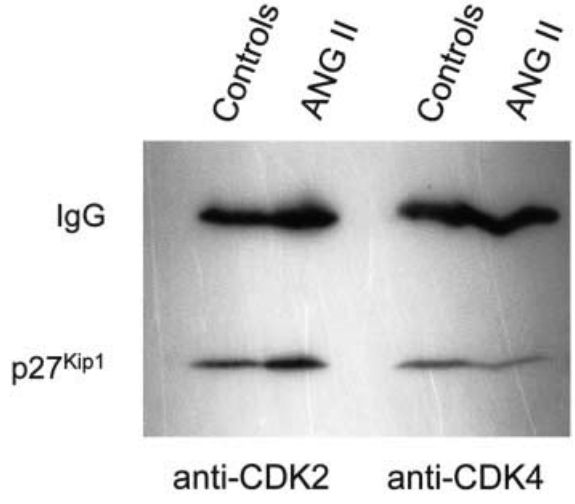

Fig. 3 Immunoprecipitation with anti-CDK antibodies with subsequent western blotting and detection of $p27^{Kip1}$. $p27^{Kip1}$ associates with CDK2 and CDK4. However, an increased binding of $p27^{Kip1}$ to CDK2 complexes was detected in tubular lysates of ANG II infused rats. There was also a small decrease in $p27^{Kip1}$ binding to CDK4 in ANG II treated animals, suggesting a potential redistribution to CDK2 containing complexes. Immunoprecipitation experiments were performed independently twice, with qualitatively similar results

However, coadministration of the antioxidant DMTU at a dose previously reported to inhibit proteinuria in a mouse model of oxygen radical mediated glomerulosclerosis [11] had no significant effect on blood pressure (Table 1). The magnitude of ANG II induced blood pressure increase was comparable to that in a previous study using similar doses of ANG II [12]. Infusion of norepinephrine increased systolic blood pressure to a similar height as ANG II (Table 1). Isolated tubules from ANG II infused rats produced significantly more O^{-}_{2} as measured by the lucigenin method than those isolated from control-infused or norepinephrine-infused rats (Table 1). Coinfusion with DMTU significantly reduced measurable O^{-}_{2} synthesis (Table 1). A representative tracing of O^{-}_{2} measurement using the lucigenin method is shown in Fig. 1.

Expression of $p27^{Kip1}$

We next tested whether ANG II infusion stimulates tubular $p27^{Kip1}$ expression similarly as in our in vitro observation with cultured tubular cells [8]. As shown in Fig. 2A, ANG II infusion significantly stimulated $p27^{Kip1}$ protein expression in isolated tubular cells as detected by western blotting. Coinfusion of DMTU completely attenuated this induction (Fig. 2A). In contrast, infusion of norepinephrine failed to increase $p27^{Kip1}$ protein expression (Fig. 2A). However, reverse transcription and cDNA amplification revealed that ANG II infusion does not stimulate an increase in $p27^{Kip1}$ transcripts (Fig. 2B; controls 1.0±0.0, ANG II infusion 1.2±0.2, relative $p27^{Kip1}$ RNA expression normalized to GAPDH, n=3, n.s.).

To determine which CDK complexes associate with the increased $p27^{Kip1}$ protein in ANG II infused rats we performed immunoprecipitation experiments using antibodies against CDK2 and CDK4 with subsequent western blotting for $p27^{Kip1}$. As shown in Fig. 3, $p27^{Kip1}$ associates with both CDK2 and CDK4. However, an increased binding of $p27^{Kip1}$ to CDK2 containing complexes was detected in tubules from ANG II infused rats (Fig. 3).

Immunohistochemical staining for $p27^{Kip1}$ revealed that nuclear expression of this CDK inhibitor is substan-

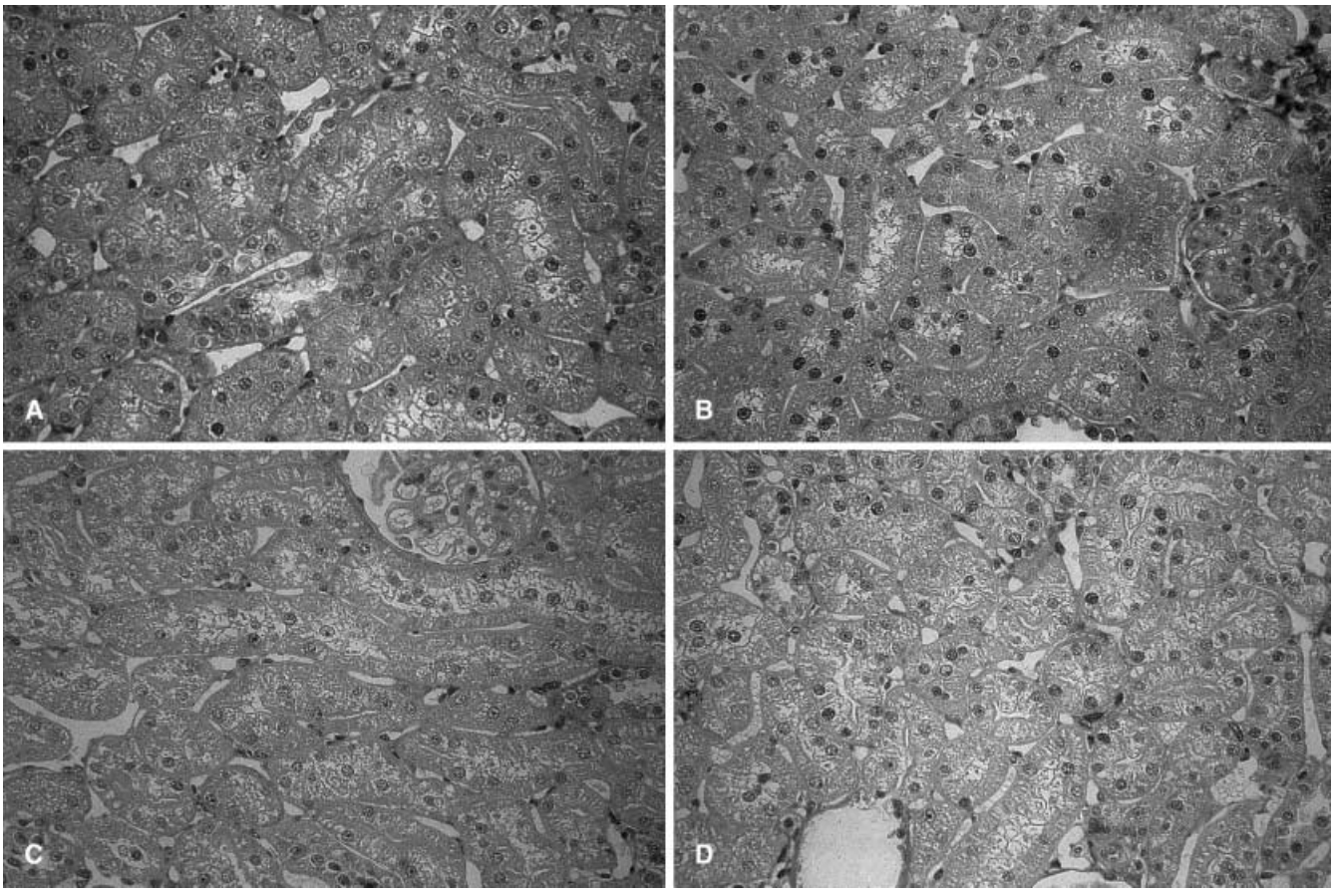

Fig. 4A–D Immunohistochemical staining for $p27^{Kip1}$. **A** Section from control-infused rat. **B** ANG II infused rat. **C** Control rats treated with DMTU. **D** Infusion of ANG II and DMTU. A clearly greater number of tubular cells staining positive for $p27^{Kip1}$ is visible in ANG II infused rats (**B**) than in controls (**A**). Cotreatment of rats with ANG II and DMTU clearly decreased the number of $p27^{Kip1}$ positive tubules (**D**)

tially higher in proximal tubular cells from ANG II infused rats than in those from animals infused with PBS only (Fig. 4A,B; Table 1). DMTU infusion abolished almost completely the ANG II induced increase in $p27^{Kip1}$ staining, suggesting mediation by reactive oxygen species (Fig. 4D; Table 1). Infusion of norepinephrine failed to significantly increase the number of tubular cells staining positive for $p27^{Kip1}$ (Table 1). Staining for the proliferation marker PCNA revealed a pattern opposite to that of $p27^{Kip1}$ (Fig. 5). Tubular PCNA expression was significantly reduced in ANG II infused rats whereas DMTU increased expression (Fig. 5; Table 1).

Discussion

This study demonstrates for the first time that infusion of ANG II into naive rats increases the tubular expression of $p27^{Kip1}$. Induction of $p27^{Kip1}$ was associated with a decrease in proliferation as assessed by immunohistochemical staining for PCNA. Induction of $p27^{Kip1}$ does not depend on hypertension per se because infusion of norepinephrine failed to induce tubular $p27^{Kip1}$ expression despite a comparable increase in systolic blood pressure. ANG II induced reactive oxygen species appear to be a mediator of the increase in tubular $p27^{Kip1}$ expression because the radical scavenger DMTU abolished the induction. This observation is in excellent agreement with previous investigations demonstrating that infusion of ANG II or pathophysiological situations with a stimulated renin-angiotensin system, but not the administration of norepinephrine, leads to stimulated O^{-}_{2} vascular production [13]. Moreover, cell cultures studies have revealed that ANG II stimulates O^{-}_{2} synthesis in renal tubular and mesangial cells [8, 13]. An ANG II mediated increase in the activity of membrane-bound NAD(P)H-oxidase, a key enzyme in one pathway of O^{-}_{2} generation, is apparently responsible for this induction (for review see [9]). It has previously been shown in cultured renal cells and in the vascular beds of infused animals that ANG II increases the activity of membrane NAD(P)H-oxidase [14]. This may be an important mechanism by which the vasopeptide stimulates production in O^{-}_{2} tubular cells. The mechanism is obviously independent of ANG II induced hypertension because norepinephrine failed to increase tubular generation of reactive oxygen products [15].

Recent studies in double-transgenic rats expressing human renin and angiotensinogen have convincingly shown that antioxidants such as pyrrolidine dithiocarba-

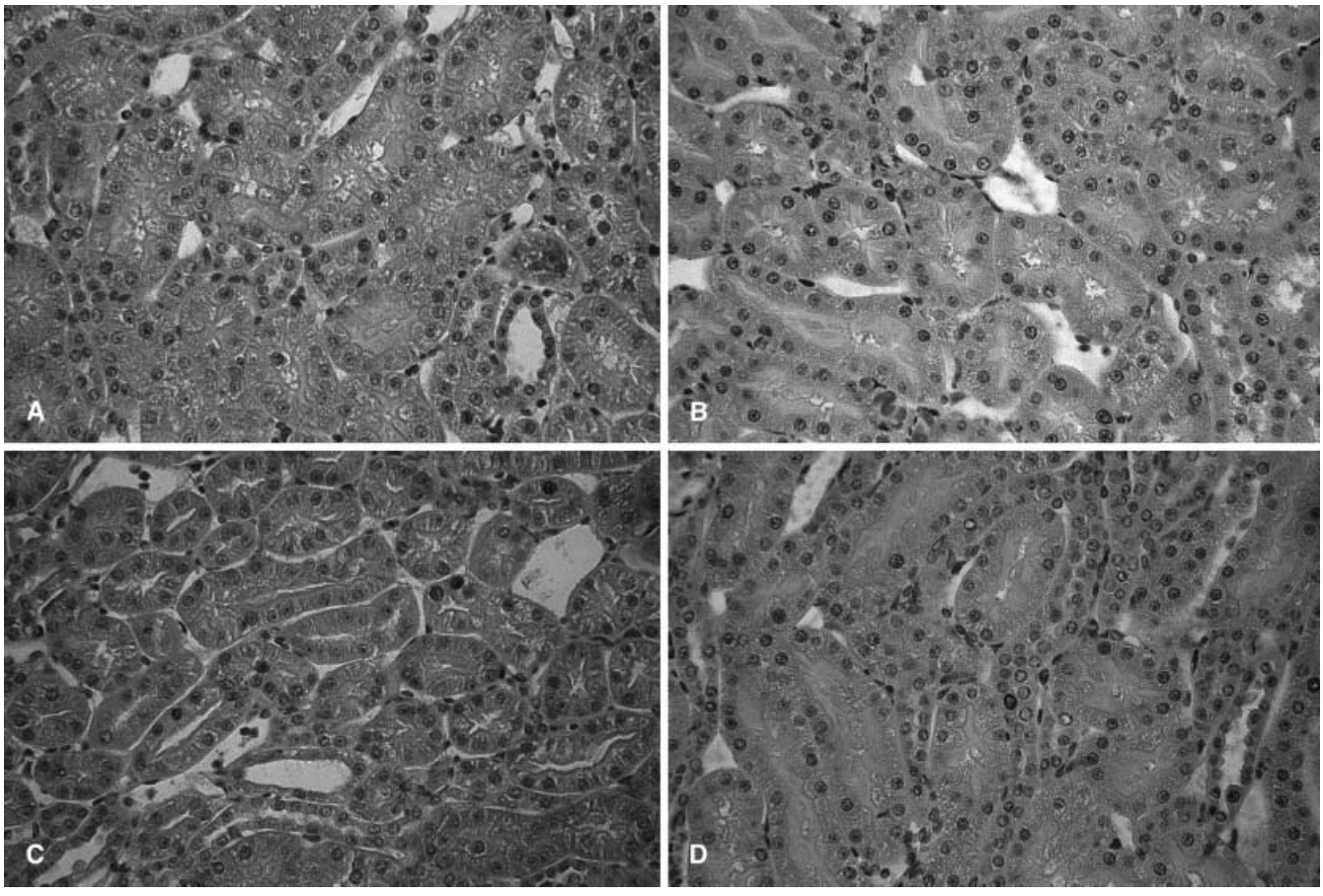

Fig. 5A–D Immunohistochemical staining for the proliferation marker PCNA. **A** Section from control-infused rat. **B** ANG II infused rat. **C** Control rats treated with DMTU. **D** Infusion of ANG II and DMTU. Only a few cells stained positive for PCNA (*arrowheads*), suggesting a relatively low turnover. ANG II infusion further reduced the number of tubular cells staining positive for PCNA (**B**) compared with controls (**A**). DMTU administration alone significantly increased PCNA staining (**C**) whereas cotreatment of rats with ANG II and DMTU attenuated the cell cycle arresting effect of ANG II (**D**)

mate also attenuate the proinflammatory effects of ANG II by preventing nuclear factor κB activation [16]. These data clearly indicate a multifactorial role of ANG II mediated oxygen radicals in renal pathology [16].

Our data suggest that the ANG II mediated local increase in O^{-}_{2} production is essential for the subsequent induction of $p27^{Kip1}$. Previous studies have demonstrated that $p27^{Kip1}$ expression is often regulated on a posttranscriptional level [17]. Moreover, more recent investigations have also found transcriptional induction of $p27^{Kip1}$ in certain systems. For example, Medema et al. [18] reported that specific transcription factors such as AFX increase $p27^{Kip1}$ mRNA. However, using a sensitive cDNA amplification assay after reverse transcription of RNA, we found in the present study no evidence for upregulation of tubular $p27^{Kip1}$ mRNA by ANG II infusion. This is essentially in agreement with previous studies from our group demonstrating that the ANG II stimulated increase in $p27^{Kip1}$ expression in cultured tubular cells in posttranscriptionally [7].

The way in which O^{-}_{2} stimulates $p27^{Kip1}$ expression is currently not completely understood. However, we have recently obtained data from cell culture studies demonstrating that reactive oxygen species stimulate phosphorylation and activation of mitogen-activated protein (MAP) kinases [19]. Such MAP kinases directly phosphorylate $p27^{Kip1}$ on serine/threonine residues and may, in contrast to tyrosine phosphorylation, increase the half-life time of this protein (unpublished data). This is in agreement with the findings of a recent study demonstrating that phosphorylation at serine residue 10 increases the protein stability of $p27^{Kip1}$ [20]. However, there are conflicting results concerning the role of MAP kinases, and other studies have reported that MAP activation stimulates degradation of $p27^{Kip1}$ [21]. One explanation for these different observations may be that phosphorylation of threonine 187 promotes degradation whereas phosphorylation on serine 10 increases $p27^{Kip1}$ stability [22]. In fact, in phosphopeptide mapping experiments after in vitro phosphorylation of $p27^{Kip1}$ by activated MAP kinases we found a much stronger phosphorylation of serine than with threonine (unpublished data). We are currently mutating the three potential serine/threonine phosphorylation sites of $p27^{Kip1}$ to further address these issues.

Although common wisdom suggests that the CDK inhibitor binds to cyclin/CDK complexes and inhibits their activity, the situation is probably much more complex, and there may be a delicate balance, with shifting of CDK inhibitors among various cyclin/CDK complexes [23, 24]. Moreover, CDK inhibitors may also have a paradoxical role in activating these kinases, particularly cyclin D/CDK4,6 complexes [23]. Immunoprecipitation experiments have demonstrated that $p27^{Kip1}$ from renal tubules of ANG II infused rats associates with CDK2. Although we have not studied whether the kinase activity of cyclin E/CDK2 complexes is actually inhibited by the increase in $p27^{Kip1}$ binding, our results are in good agreement with the observations of Servant et al. [25], who reported inhibition of CDK2 by increased $p27^{Kip1}$ levels in ANG II mediated hypertrophy of cultured aortic smooth muscle cells.

Cell culture experiments clearly suggest that the increase in $p27^{Kip1}$ is necessary for ANG II induced hypertrophy [8, 19]. However, the present study did not measure whether infusion of ANG II indeed leads to tubular hypertrophy. Longer infusion periods of ANG II are probably necessary for this effect. In addition, it is intrinsically difficult to quantify tubular hypertrophy in vivo. Nevertheless, we will try in future studies to address these issues.

ANG II mediated effects on $p27^{Kip1}$ expression likely depend on the organ studied. A recent study, published after submission of our manuscript, revealed that subcutaneously infusion in a somewhat lower dose for 7 days down-regulated $p27^{Kip1}$ expression in mesenteric vessels [26]. This attenuated $p27^{Kip1}$ expression was associated with increased proliferation [26]. However, in vitro studies in cultured vascular smooth muscle cells demonstrate that ANG II induces $p27^{Kip1}$ and stimulates hypertrophy, similar to our observations [27].

Previous studies revealed that a diet deficient in vitamin E and selenium induces glomerular and tubular hypertrophy in naive rats, suggesting that reactive oxygen species modulate renal growth in vivo [28]. Since tubular hypertrophy is likely an early marker of subsequent development of chronic irreversible renal injury such as interstitial fibrosis [1, 2], therapeutic manipulation of this adaptive growth process may prevent deterioration of renal function in chronic renal disease. Since local ANG II levels are elevated in almost all chronic renal diseases, it is intriguing to hypothesize that early administration of oxygen scavengers may be prevent tubular induction of $p27^{Kip1}$, cell cycle arrest, and the development of interstitial fibrosis.

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