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Angiotensin converting-enzyme inhibitor treatment reduces glomerular $p16^{INK4}$ and $p27^{Kip1}$ expression in diabetic *BBdp* rats

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Abstract

Aims/hypothesis. Renal hypertrophy occurs early in diabetes mellitus and precedes the development of glomerulosclerosis and tubulointerstitial fibrosis. We have previously shown that cultured mesangial cells exposed to high glucose are arrested in the G_1-phase of the cell cycle and undergo cellular hypertrophy. High glucose-mediated induction of $p27^{Kip1}$, an inhibitor of cyclin-dependent kinases, is essential in this process. Further investigations have also shown that $p27^{Kip1}$ and $p21^{Cip1}$, other cyclin-dependent kinase inhibitors, are up regulated in the kidneys of mice with Type I (insulin-dependent) as well as Type II (non-insulin-dependent) diabetes mellitus. Our study was undertaken to test a potential effect of short-term treatment with the angiotensin-converting enzyme inhibitor enalapril on the glomerular expression of the cyclin-dependent kinase inhibitors $p16^{INK4}$, $p21^{Cip1}$, and $p27^{Kip1}$ in *BBdp* rats, an autoimmune model of Type I diabetes.
Methods. We evaluated $p16^{INK4}$, $p21^{Cip1}$, and $p27^{Kip1}$ protein expression in isolated glomeruli by western blots. We also assessed $p27^{Kip1}$ positive glomerular cells by immunohistochemistry.
Results. Glomerular expression of all three cyclin-dependent kinase inhibitors were stimulated in *BBdp* rats compared with non-diabetic *BBdr* animals. Enalapril treatment for 3 weeks, started after the onset of diabetes, reduced the glomerular expression of $p16^{INK4}$ and $p27^{Kip1}$ but not of $p21^{Cip1}$. Enalapril also prevented the increase in kidney weights observed in *BBdp* rats but had no effect on systolic blood pressure or glucose concentrations.
Conclusion/interpretation. Our data show that enalapril attenuates the glomerular expression of cyclin-dependent kinase inhibitors in diabetes and suggest a molecular mechanism of how angiotensin-converting enzyme inhibitors prevent renal hypertrophy in diabetes. [Diabetologia (1999) 42: 1425–1432]

Keywords Diabetic nephropathy, cyclin-dependent kinase inhibitors, glomerular hypertrophy, cell cycle arrest, angiotensin II, progression of renal failure.

Diabetic nephropathy encompasses several structural alterations including renal hypertrophy, thickening of basement membranes and progressive glomerular accumulation of extracellular matrix components [1–3]. Expansion of the glomerular mesangium occurs within weeks of the onset of experimental Type I (insulin-dependent) diabetes mellitus and correlates closely with the deterioration in glomerular function [1–3]. Cell culture studies using mesangial cells exposed to high glucose concentration medium as well as in vivo investigations in various models of diabetes suggest a biphasic growth response: there is a very early and self-limited proliferation of mesangial cells with subsequent cell cycle arrest in the G_1-phase and concomitant hypertrophy [4–6]. We and others have previ-

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Abbreviations: Cdk, Cyclin-dependent kinases; ACE, angiotensin converting-enzyme; ANG II, angiotensin II; RAS, renin-angiotensin system.

ously described that this G_1-phase arrest is principally mediated by inhibitors of cyclin-dependent kinases (Cdk) such as $p27^{Kip1}$ and $p21^{Cip1}$ [7–9]. High glucose stimulates expression of these small proteins [7]. Because angiotensin converting enzyme (ACE) inhibitors prevent glomerular hypertrophy and slow progression of diabetic nephropathy in many models as well as in humans suffering from diabetes [10–14], our study was undertaken to test a potential influence of the ACE inhibitor enalapril on the glomerular expression of cyclin-dependent kinase inhibitors in *BBdp* rats, an animal model of spontaneous Type I diabetes [15].

Material and methods

Rats. A breeding colony of *BBdp* and *BBdr* rats was established in our animal facility. Because the onset of diabetes is sudden in *BBdp* rats and occurs at about 60–100 days of age [15–19], urine was screened daily by dip sticks starting at age 55 days. If the urine showed a positive reaction for glucose for 3 consecutive days, blood was drawn from the tail to confirm hyperglycaemia. Blood glucose concentrations were measured with B-glucose analyser (HemoCue, Ängelholm, Sweden). Hyperglycaemic *BBdp* rats received subcutaneously a sustained release insulin implant (Lindplant, Linshin, Ontario, Canada; releases 2 units insulin per 24 h). This treatment prevents the development of overt ketosis and death but does not render the animals normoglycaemic. To control for potential confounding sex effects, only male rats were used. After onset of diabetes, *BBdp* rats were allocated at random into two groups either receiving normal tap water or water supplemented with 100 mg enalapril (Sigma, Deisenhofen, Germany) per litre water for 3 weeks. Age-matched *BBdr* rats which do not develop diabetes were treated for 3 weeks with tap water and served as controls [19]. Water intake for *BBdp* rats was approximately 70 ml/day. Thus, these animals received roughly 23 mg enalapril per kg body weight per day. Non-diabetic *BBdr* rats drank 30 ml water per day. After 3 weeks, systolic blood pressure was measured in slightly restrained rats by tail plethysmography. Animals were weighed and anaesthetized. Blood was drawn by puncture of the aorta, kidneys were removed, wet weight was determined and kidneys were kept on ice until further processing. A slice of each kidney was fixed in Methyl Carnoy's solution for immunohistochemistry. All animal experiments were approved by the local government and were done according to the "principles of laboratory animal care" (NIH publication No. 86–23, 1985). Five to eight animals were investigated in each group.

Western blots. Glomeruli were separately isolated from each animal by differential sieving using techniques established for this laboratory [8]. Final preparations contained more than 95 % glomeruli as judged by light microscopy. Isolated glomeruli were directly lysed on ice in 300 µl of a buffer containing 2 % SDS and 60 mmol/l TRIS-HCl (pH 6.8) supplemented with a cocktail of protease inhibitors (Complete, Boehringer Mannheim, Germany; contains antipain-HCl, chymostatin, leupeptin, bestatin, pepstatin, phosphoramidon, aprotinin, EDTA). After centrifugation, the supernatants were transferred to new tubes and protein contents were measured by a modification of the Lowry method. Protein concentrations were adjusted to 60 µg/sample and 5 % glycerol/0.03 % bromophenol blue/10 mmol/l dithiothreitol were added, then samples were boiled for 5 min. After centrifugation, supernatants were loaded onto a denaturing 8 % SDS polyacrylamide gel. Low molecular weight markers (Rainbow markers; Amersham, Braunschweig, Germany), which comprise 2350 to 45 000 Daltons served as the molecular weight standards. After completion of electrophoresis, proteins were electroblotted onto a nitrocellulose membrane (High-bond-N, Amersham) in transfer buffer (50 mmol/l TRIS-HCl [pH 7.0], 380 mmol/l glycine, 0.1 % SDS, 20 % methanol). Filters were stained with Ponceau S to control for equal loading and transfer. The blots were blocked in 5 % non-fat dry milk in PBS with 0.1 % Tween 20 for 1 h at 22 °C. For the detection of $p27^{Kip1}$, a mouse monoclonal anti-human $p27^{Kip1}$ antibody was obtained from Transduction Laboratories (Lexington, Mass., USA). This antibody reacts with rat $p27^{Kip1}$. The $p21^{Cip1}$ protein expression was detected with a mouse monoclonal anti-human $p21^{Cip1}$ antibody having cross-reactivity with the murine protein (Dako, Glostrup, Denmark). These primary antibodies were used in a 1:1000 dilution. The $p16^{INK4}$ was detected with mouse anti-human $p16^{INK4}$ monoclonal antibody (Pharmingen, Hamburg, Germany). The $p16^{INK4}$ antibody was used in a 1:500 dilution. Although this antibody has not been previously examined for cross-reactivity, it reacted with a 16 kDa protein in our experiments suggesting that this antibody detects rat $p16^{INK4}$. To further test the specificity of this antibody, a 1:200 dilution of this antibody was incubated with 10 µg of recombinant human $p16^{INK4}$ GST fusion protein (Pharmingen) for 30 min. After centrifugation, the supernatant was used for western blot detection and showed no specific binding to the 16 kDa rat protein anymore suggesting specific depletion by the exogenous antigen (data not shown). Washes, incubations with horseradish peroxidase-conjugated anti-mouse secondary antibodies and detection using the ECL reagent (Amersham) were done according to the manufacturer's recommendations. To control for small variations in protein loading and transfer, membranes were washed and reincubated with a mouse monoclonal anti-β-actin antibody (Sigma). Incubation with secondary antibody and detection was done as described above. Exposed films were scanned with Fluor-S multi-imager (Bio-Rad laboratories, Hercules, USA) and data were analysed with the computer program Multi-Analyst from Bio-Rad. A ratio of the intensities of the bands for $p16^{INK4}$, $p27^{Kip1}$, $p21^{Cip1}$ and β-actin was calculated [7, 8]. Western blots were independently repeated three times with qualitatively similar results.

Immunohistochemistry. We prepared two µm thick paraffin sections. To show the immunohistochemistry 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. As additional controls, slides were incubated with normal mouse serum. The alkaline phosphatase-antialkaline phosphatase (APAAP) complex was used to make the primary antibody visible as previously described [8]. Evaluation of $p27^{Kip1}$-positive cells was done by an investigator blinded to the experimental protocol. Glomerular cells expressing $p27^{Kip1}$ were counted in at least 15 glomeruli from each rat.

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

Table 1. Body weights, kidney weights, blood glucose and systolic blood pressure

	BBdr	*BBdp*	*BBdp* + Enalapril
Body weight (g)	309 ± 8	313 ± 8	303 ± 11
Left kidney weight (g)	1.00 ± 0.05	1.36 ± 0.03[b]	1.23 ± 0.04[c]
Blood glucose (mmol/l)	7 ± 0.9	18.8 ± 1.5[a]	19.9 ± 2.1[a]
Systolic blood pressure (mm Hg)	107 ± 4	109 ± 6	98 ± 4

$n = 5$–8, [a] $p < 0.01$ vs *BBdr*, [b] $p < 0.001$ vs *BBdr*, [c] $p < 0.01$ vs *BBdp*

Results

Table 1 shows body weight, renal weights, blood glucose concentrations and systolic blood pressure at the end of the experimental protocol after 3 weeks. Although *BBdp* rats and *BBdp* rats treated with enalapril received insulin pellets to prevent ketoacidosis and premature death, blood glucose concentrations were still considerably higher compared with non-diabetic *BBdr* animals. Enalapril had no influence of blood glucose concentrations. Diabetic *BBdp* rats had a higher kidney weight than the *BBdr* group. Enalapril treatment reduced this increase in weight. In accordance with previous studies, diabetic *BBdp* animals had a similar systolic blood pressure as non-diabetic *BBdr* rats [15, 16]. Although enalapril treatment numerically reduced systolic blood pressure in *BBdp* rats compared with untreated animals, these values failed to reach statistical significance. (Table 1).

Figure 1 shows a western blot of glomerular lysates incubated with an antibody against $p27^{Kip1}$. The blot was re-incubated with an antibody against β-actin to control for small variations in protein loading and transfer. Glomeruli obtained from non-diabetic *BBdr* rats had almost no expression of $p27^{Kip1}$ protein. In contrast, *BBdp* rats exhibited a significantly higher expression of $p27^{Kip1}$, although there was some variation between individual rats (*BBdr*: 1.00 ± 0.05, *BBdp*: 3.72 ± 0.2 relative changes in $p27^{Kip1}$ expression normalised to β-actin, $p < 0.01$, $n = 5$). Enalapril treatment of *BBdp* rats significantly reduced glomerular $p27^{Kip1}$ protein expression compared with the untreated group (*BBdp* + enalapril: 0.89 ± 0.04 relative changes in $p27^{Kip1}$ expression normalised to β-actin, $p < 0.01$ vs untreated *BBdp* rats, $n = 5$). Expression of $p21^{Cip1}$ was differentially regulated (Fig. 2). Although there was a greater variation of glomerular $p21^{Cip1}$ expression among individual animals with one *BBdr* rat having a high concentration and two diabetic *BBdp* animals with rather low expression, overall expression was also significantly higher in diabetic rats (*BBdr*: 1.00 ± 0.02, *BBdp*: 5.0 ± 0.1 relative changes in $p21^{Cip1}$ expression normalised to β-actin, $p < 0.05$, $n = 5$). Enalapril treatment failed, however, to reduce glomerular $p21^{Cip1}$ expression in *BBdp* rats (*BBdp* + enalapril: 4.5 ± 0.1 relative changes in $p21^{Cip1}$ expression normalized to β-actin, not significant vs untreated *BBdp* rats, $n = 5$). Base line $p16^{INK4}$ expression in non-diabetic *BBdr* rats was somewhat higher compared with $p27^{Kip1}$ and $p21^{Cip1}$ concentrations (Fig. 3). Glomerular $p16^{INK4}$ expression was significantly increased in *BBdp* rats and was attenuated by enalapril treatment (Fig. 3; *BBdr*: 1.0 ± 0.4, *BBdp*: 5.1 ± 0.9*, *BBdp* + enalapril: $1.6 \pm 0.2^{+}$ relative changes in $p16^{INK4}$ expression normalised to β-actin, $^{*}p < 0.01$ vs *BBdr*, $^{+}p < 0.01$ vs *BBdp*, $n = 6$ for *BBdr*, $n = 8$ for *BBdp*, $n = 4$ for *BBdp* + enalapril).

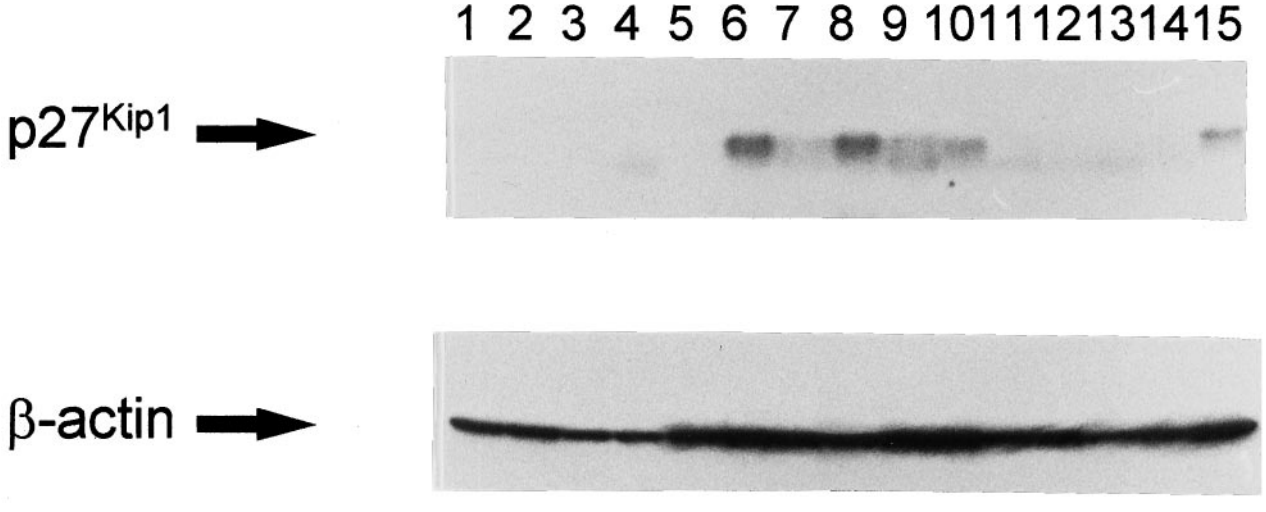


Fig. 1. Western blot of glomerular lysates incubated with an antibody against $p27^{Kip1}$. Each lane represents glomerular protein from an individual animal. The blot was washed and reincubated with an antibody against β-actin to account for small variations in protein loading and transfer. This blot is representative of three experiments with qualitatively similar results. Lanes 1–5: non-diabetic *BBdr* rats, lanes 6–10: diabetic *BBdp* rats, lane 11–15: diabetic *BBdp* after receiving enalapril for 3 weeks

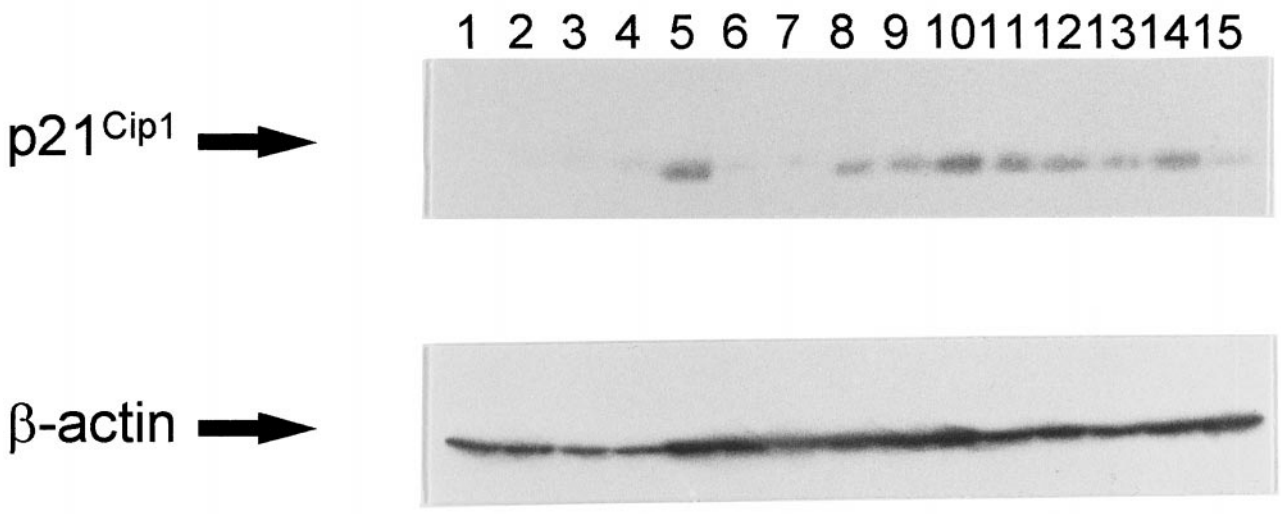


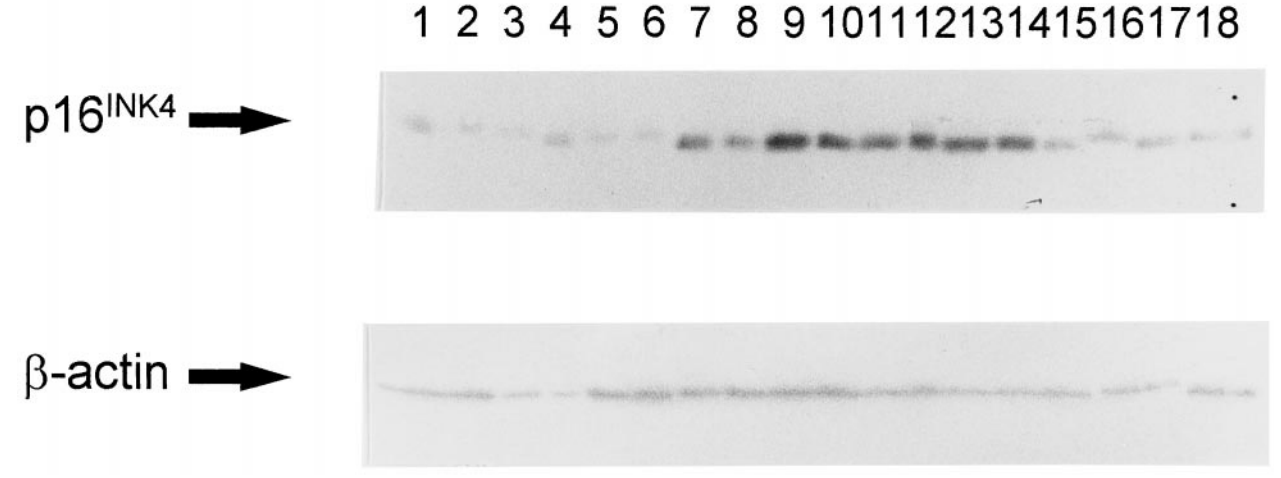
Fig. 2. Western blot for $p21^{Cip1}$. This blot is representative of three experiments with qualitatively similar results. Lanes 1–5: non-diabetic *BBdr* rats, lanes 6–10: diabetic *BBdp* rats, lane 11–15: diabetic *BBdp* after receiving enalapril for 3 weeks

To further confirm that enalapril attenuates glomerular $p27^{Kip1}$ expression, immunohistochemistry staining was done and positive cells were counted by an investigator blinded to the experimental protocol. Because the majority of cells expressing $p27^{Kip1}$ in the normal rat glomerulus are podocytes [8, 20] and it can be occasionally difficult to unambiguously decide to what exact type a positive glomerular cell belongs, we counted all glomerular cells expressing $p27^{Kip1}$. As shown in Fig. 4 A-C, the number of total glomerular cells expressing $p27^{Kip1}$ was significantly increased in *BBdp* rats compared with *BBdr* animals (*BBdr*: 18.7 ± 1.2, *BBdp*: 35.0 ± 2.5 positive cells per glomerulus, $p < 0.001$, at least 15 glomeruli counted from each rat, $n = 5$). Enalapril treatment of *BBdp* rats significantly reduced the glomerular number of $p27^{Kip1}$ positive cells (*BBdp* + enalapril: 21.0 ± 1.6 positive cells per glomerulus, $p < 0.001$ vs untreated *BBdp* rats, at least 15 glomeruli counted from each rat, $n = 5$).

Fig. 3. Western blot for $p16^{INK4}$. This blot is representative of three experiments with qualitatively similar results. Lanes 1–6: non-diabetic *BBdr* rats, lanes 7–14: diabetic *BBdp* rats, lane 15–18: diabetic *BBdp* after receiving enalapril for 3 weeks

Discussion

Renal hypertrophy occurs early after the onset of diabetes mellitus and precedes the development of glomerulosclerosis [1, 2, 21]. Experiments in different animal models of diabetes as well as studies in humans have provided convincing evidence that ACE-inhibitor treatment could prevent and even partly reverse renal hypertrophy [10–13]. Although a restoration to normal of glomerular hyperfiltration, which possibly activates through mechanical stretch mesangial and endothelial growth processes, could account for this effect, there is increasing evidence that angiotensin II (ANG II) exerts direct growth stimulatory effects on glomerular cells which could be further en-



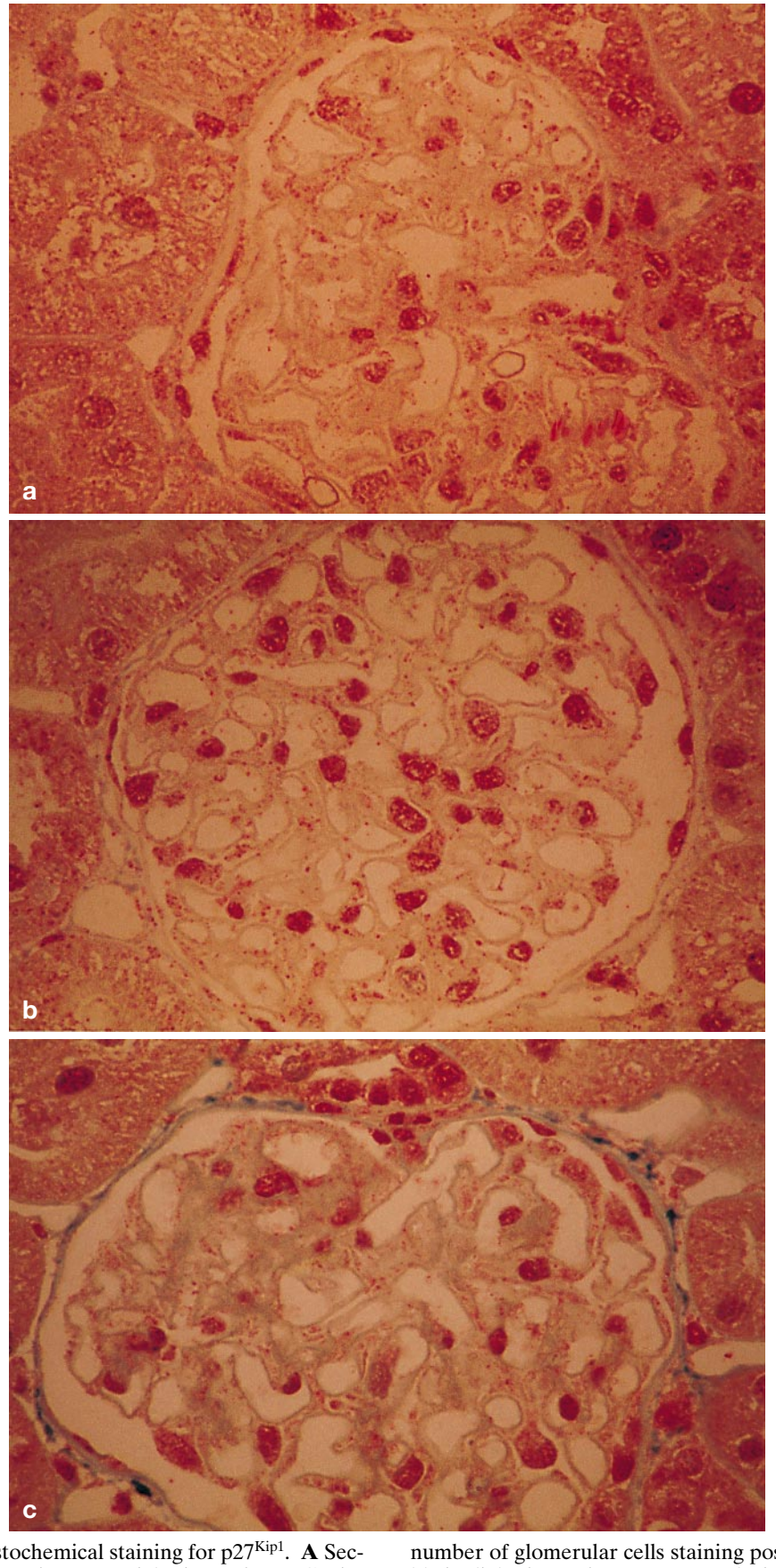


Fig. 4 A–C. Immunohistochemical staining for $p27^{Kip1}$. **A** Section from non-diabetic *BBdr* rat, **B** Diabetic *BBdp* rat, **C** Diabetic rat after enalapril treatment. A clear increased in the number of glomerular cells staining positive for $p27^{Kip1}$ is visible in diabetic *BBdp* rats compared with the *BBdr* littermates. Enalapril treatment reduces this increase

hanced in the diabetic milieu [22–25]. High glucose as well as advanced glycation end products induce in vitro and in vivo transforming growth factor beta (TGF-β) in the kidney [5, 22, 23]. Neutralization experiments have clearly shown that TGF-β is a necessary prerequisite for the development of glomerular hypertrophy in streptozotocin-induced diabetic mice [23]. Angiotensin II also induces the expression of TGF-β in renal cells and the effects of high glucose concentration and ANG II on hypertrophy of cultured renal cells are additive [24–27].

The molecular mechanisms of how high glucose concentration as well as ANG II induce cellular hypertrophy have, however, been more recently explained. Kinase activity of cyclin-Cdk complexes is essential for driving cells through the cell cycle [28]. Cyclin-Cdk complexes are negatively regulated by small proteins called Cdk inhibitors. Principally, two different families of Cdk inhibitors can be distinguished based on the target cyclin-Cdk they inhibit and on shared homologous sequences [28]. These small proteins bind to cyclin-Cdk complexes and inhibit their kinase activity. The consequence is G_1-phase arrest and the cell can undergo hypertrophy.

High glucose concentration induces the Cdk inhibitors $p27^{Kip1}$ and $p21^{Cip1}$ in cultured mesangial cell [7–9]. These Cdk inhibitors are also expressed in mesangial cells in vivo in different animal models of diabetes [7–9]. Because glomerular podocytes constitutively express Cdk inhibitors [20], it is occasionally difficult to unambiguously decide whether glomerular cells staining positive for $p27^{Kip1}$ are indeed of mesangial origin. Therefore, we decided to count all positive glomerular cells and expression was clearly increased in diabetic *BBdp* rats and attenuated by enalapril treatment. Overexpression of Cdk inhibitors such as $p16^{INK4}$ and $p21^{Cip1}$ reduces proliferation but stimulates cellular hypertrophy of various cultured renal cells [29]. Thus, induction of Cdk inhibitors appears to be the pivotal underlying molecular mechanism for the development of cell hypertrophy.

Enalapril clearly reduced in our study the increased kidney weights in *BBdp* rats to values of non-diabetic *BBdr* controls. Although total kidney weight is only a rather crude measure of hypertrophy, these findings provide a correlation between compensatory hypertrophy and the expression of $p16^{INK4}$ and $p27^{Kip1}$ in the enlarging kidney.

Although we have previously shown in cultured mesangial cells that high glucose concentration alone is sufficient to stimulate $p27^{Kip1}$ expression [7], enalapril did not reduce hyperglycaemia in *BBdp* rats suggesting that other mechanisms than a simple decrease in blood glucose concentration must underlie the enalapril-mediated reduction in $p27^{Kip1}$ positive cells. In accordance with previous investigations, diabetic *BBdp* rats are not hypertensive compared with their non-diabetic littermates [16–19]. Thus, it is unlikely that an enalapril-mediated reduction in systemic blood pressure contributed to the decrease in glomerular $p27^{Kip1}$ expression. *BBdp* rats show glomerular hyperfiltration [18] and we cannot therefore rule out that a normal in glomerular filtration rate caused by the ACE inhibitor is involved in attenuated $p27^{Kip1}$ expression. We would, however, like to propose a more direct effect of ANG II in the induction of $p27^{Kip1}$ expression. *BBdp* rats have an activated renin-angiotensin system (RAS) because it has been previously shown that renin secretion is stimulated in this rat strain [16]. Furthermore, an increase in urinary endothelin excretion could additionally provide an indirect hint to an activated RAS because ANG II stimulates renal endothelin synthesis [30]. We have previously shown in proximal tubular cells that ANG II stimulates expression of $p27^{Kip1}$ and that this induction is associated with G_1-phase arrest and cellular hypertrophy [31]. Intracellular reactive oxygen species, produced by an ANG II-mediated induction of membrane NADPH oxidase activity, are important signalling intermediates in the increase in $p27^{Kip1}$ expression [32]. We have preliminary evidence that oxygen radicals activate mitogen-activated protein (MAP) kinases which in turn directly phosphorylate $p27^{Kip1}$ leading to an increase in expression (Hannken and Wolf, unpublished observations). Because the intrarenal RAS is activated in *BBdp* rats, it is possible that ANG II directly stimulates $p27^{Kip1}$ expression. Enalapril interferes with ANG II generation, explaining the attenuation of $p27^{Kip1}$ expression. Increased oxidative stress is enhanced in the diabetic environment [33] and could further stimulate $p27^{Kip1}$ expression. Notably, it has been reported that enalaprilat has direct antioxidant effects in cultured mesangial cells and inhibits hydrogen peroxide formation induced by high glucose concentrations in these cells [34]. This action is not linked to ACE inhibition [34]. Thus, enalapril could interfere with oxygen radical formation and prevent by this mechanism the induction of $p27^{Kip1}$.

Notably, $p21^{Cip1}$ expression, albeit previously shown to be stimulated in diabetic glomeruli [9], was not reduced by enalapril treatment. It has been previously shown that $p21^{Cip1}$, in contrast to $p27^{Kip1}$, can be transcriptionally induced by a p53-dependent mechanism [35]. One possible explanation for the differential regulation could be that $p21^{Cip1}$ expression depends more on the diabetic environment such as ambient high glucose concentration and not so much on a locally activated RAS.

The $p16^{INK4}$ is the prototype of the INK4 family of Cdk inhibitors and members of this family differ in both structure and mechanism of action. In contrast to $p21^{Cip1}$ and $p27^{Kip1}$ which both bind to and inhibit the kinase activity of almost all different Cdks, $p16^{INK4}$ is much more selective in its inhibitory activities, affecting only Cdk4 and Cdk6 [36]. Members of

the INK4 family contain four or more ankyrin repeats and $p16^{INK4}$ is a tumour suppressor gene [37, 38]. Previous studies in non-renal cells have shown that TGF-β-mediated induction of INK4 members block activation of cyclin D-Cdk4 complexes by displacement of $p27^{Kip1}$ from these complexes to downstream binding and inhibition of cyclin E-Cdk2 heterodimers [38]. Thus, there could be a close functional interaction between $p16^{INK4}$ and $p27^{Kip1}$ explaining why enalapril influences the expression of both Cdk inhibitors. We did not, however, test whether other treatments which also interfere with glomerular haemodynamics such as calcium antagonists influence $p27^{Kip1}$ expression.

Several observations convincingly indicate that the development of irreversible glomerulosclerosis in diabetes is always preceded by the early renal hypertrophy [39]. It is debatable whether or not these hypertrophic processes will inevitably lead to irreversible fibrotic changes in humans but experimental animal models provide ample evidence that this is the case [39]. Treatment with ACE inhibitor prevents kidney hypertrophy and slows the progression of renal disease.

In summary, short-term ACE-inhibitor treatment for 3 weeks prevented compensatory renal hypertrophy and the stimulated glomerular expression of the Cdk-inhibitors, $p16^{INK4}$ and $p27^{Kip1}$ in *BBdp* rats, an autoimmune model of Type I diabetes. Although $p21^{Cip1}$ was also increased in glomeruli of diabetic animals, enalapril failed to reduce the expression of this Cdk inhibitor. Our results provide an insight into the molecular mechanism of how ACE inhibitors possibly modify renal hypertrophy and slow the progression of renal disease in diabetes.

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