Hypertension Induces Somatic Cellular Senescence in Rats and Humans by Induction of Cell Cycle Inhibitor p16\textsuperscript{INK4a}

Jens H. Westhoff, Karl F. Hilgers, Mario P. Steinbach, Andrea Hartner, Bernd Klanke, Kerstin Amann, Anette Melk

Abstract—There is increasing evidence for a role of somatic cellular senescence in physiological aging but also in injury and disease. Cell cycle inhibitor p16\textsuperscript{INK4a} is the key mediator for stress and aberrant signaling induced senescence. Here we report that elevated blood pressure markedly induced p16\textsuperscript{INK4a} expression in rat kidneys and hearts, as well as in human kidneys. In kidneys from deoxycorticosterone acetate-salt–treated rats, p16\textsuperscript{INK4a} induction was found in tubular, glomerular, interstitial, and vascular cells and correlated with the typical histopathologic features of hypertensive target organ damage. p16\textsuperscript{INK4a} expression also correlated with phospho-p38, a positive upstream regulator of p16\textsuperscript{INK4a} expression. In left ventricles, increased p16\textsuperscript{INK4a} expression was found in myocardium and cardiac arteries. Antihypertensive medication consistent of hydrochlorothiazide, hydralazine, and reserpine ameliorated the histopathologic changes and attenuated p16\textsuperscript{INK4a} expression in kidneys of deoxycorticosterone acetate-salt–treated rats. Nonantihypertensive administration of spironolactone also reduced kidney damage and p16\textsuperscript{INK4a} expression. p16\textsuperscript{INK4a} induction was further observed in kidneys from hypertensive transgenic rats heterozygous for the mouse Ren-2 gene and was prevented by the angiotensin II type 1 receptor blocker losartan. In human kidney biopsies showing hypertensive nephrosclerosis, increased p16\textsuperscript{INK4a} expression was found compared with age-matched normotensive control subjects. Thus, hypertension induces cellular senescence via p16\textsuperscript{INK4a}, possibly through p38, thereby contributing to hypertensive target organ damage. This detrimental effect can be overcome by different therapeutic drug strategies. (Hypertension. 2008;52:123-129.)

Key Words: target organ damage ■ kidney ■ heart ■ senescence ■ p16\textsuperscript{INK4a} expression ■ cell cycle inhibitor

Cellular senescence describes a state of permanent and irreversible cell cycle arrest with a reduced capability to respond to stresses that results in insufficient regenerative capacity of organs.\textsuperscript{1,2} Two major pathways have been identified to induce senescence: stress and aberrant signaling induced senescence and replicative senescence. Replicative senescence is found in humans but not in rodents and results from dysfunctional telomeres.\textsuperscript{3} Stress and aberrant signaling induced senescence is observed in both humans and rodents and can be induced by a variety of extrinsic stresses in culture\textsuperscript{4} with the upregulation of the cyclin-dependent kinase inhibitor p16\textsuperscript{INK4a} as a key feature. p16\textsuperscript{INK4a} binds to CDK4 and inhibits its interaction with cyclin D, thereby preventing the passage through the G1 phase of the cell cycle.\textsuperscript{5}

There is increasing evidence for a role of cellular senescence in the aging of mammalian organisms.\textsuperscript{1} p16\textsuperscript{INK4a} induction is a unique feature of renal aging and was also demonstrated in native kidney diseases and transplantation-associated diseases.\textsuperscript{6–8} Common histopathologic features of renal aging and renal diseases, such as tubular atrophy, interstitial fibrosis, and glomerulosclerosis, correlate with p16\textsuperscript{INK4a} expression.\textsuperscript{9–11} p16\textsuperscript{INK4a} induction is also found in aging rodent\textsuperscript{6,12} and human\textsuperscript{13} hearts.

Greater rates of telomere shortening and a higher incidence of senescence-associated cellular phenotypes have been associated with hypertension and the development of atherosclerosis.\textsuperscript{14} Thus, cellular senescence may be responsible for the development of typical age-related phenotypes and contributes to the decline in the regenerative capacity of the kidney, heart, and other organs with age. In contrast, accelerated aging phenotypes are seen in diseases that induce cellular senescence.

The primary objective of the present study was to investigate whether high blood pressure (BP) leads to acceleration in renal senescence features in rodents and humans and whether different therapeutic strategies have any modulating effects. Because of the existence of stress and aberrant signaling induced senescence in both human and rodent renal senescence we have focused on studying p16\textsuperscript{INK4a} expression as a central signaling protein in this senescence pathway.\textsuperscript{9,10} Secondary objectives were senescence changes to the myocardium and the importance of the renin-angiotensin-aldosterone system independent from BP.

Materials and Methods
For an expanded Material and Methods section, please see the data supplement available online at http://hyper.ahajournals.org.
The experimental protocol has been published elsewhere.16 At the angiotensin II type 1 (AT1) receptor blocker losartan for 4 weeks. Dawley controls (n/H110057) were used. Five of the TGRs received previously.9

Total RNA was extracted from renal tissue samples. After reverse transcription, quantitative PCR was performed as described previously.19

Transgenic(mRen2)27 Hypertensive Rats

Transgenic(mRen2)27 hypertensive rats (TGRs) heterozygous for the mouse Ren-2 gene are regarded as a high (tissue-) renin model (hydrochlorothiazide, hydralazine, and reserpine [DOCA-TRP]) and spironolactone in a nonantihypertensive dose (DOCA+spirono). Animals were euthanized after 4 weeks (DOCA 4 weeks, n=6) and 6 weeks (controls, n=8; DOCA 6 weeks, n=14; DOCA+TRP, n=8; DOCA+spirono, n=7). Twenty-four-hour urine was collected before sacrifice. At the day of sacrifice, intra-arterial BP was measured in conscious rats.

Mean arterial pressure increased significantly after 4 and 6 weeks of DOCA administration. Antihypertensive triple therapy but not spironolactone significantly lowered BP compared with DOCA at 6 weeks. The marked proteinuria in these rats was affected in a similar manner (Table S1).  

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### Table. Degree of Tubular Deterioration, Glomerulosclerosis, Interstitial Fibrosis, and Vascular Damage Score in DOCA-Salt Hypertensive Rats After 4 and 6 Weeks and the Influence of DOCA+TRP and DOCA+Spirono Therapy

<table>
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<tr>
<th>Groups</th>
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<tr>
<td>Atrophic tubules, %</td>
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<td>17.47±10.91</td>
<td>2.42±3.66†</td>
<td>10.57±8.99</td>
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<tr>
<td>Dilated tubules, %</td>
<td>0.18±0.47</td>
<td>1.46±1.56</td>
<td>14.47±11.95‡</td>
<td>1.75±2.67†</td>
<td>2.52±2.80‡</td>
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<td>GS index (0 to 4)</td>
<td>0.41±0.15</td>
<td>1.24±0.20‡</td>
<td>1.99±0.42*</td>
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<tr>
<td>Fibrosis index (0 to 4)</td>
<td>0.16±0.09</td>
<td>0.65±0.54</td>
<td>1.20±0.49*</td>
<td>0.59±0.19§</td>
<td>0.97±0.36</td>
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<td>VDS (0 to 4)</td>
<td>0.92±0.14</td>
<td>1.10±0.13</td>
<td>1.49±0.20*</td>
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<td>1.29±0.36</td>
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All of the values are given as means±SDs. VDS indicates vascular damage score; GS, glomerulosclerosis.

### Immunohistochemistry for p16INK4a and Phospho-p38

Immunoperoxidase staining for p16INK4a and phospho-p38 (p-p38) was performed using paraffin-embedded tissue, as described for p16INK4a previously.9 For p-p38, we used a p-p38 mitogen-activated protein kinase (MAPK, Thr180/Tyr182) antibody (12F8, rabbit monoclonal antibody, Cell Signaling Technology, Inc, Beverly, Mass). A similar protocol was used for all of the sections; additional antigen retrieval was necessary for p38 staining.

The percentage of positive nuclei was assessed for tubules, glomeruli, interstitium, and renal arteries from kidneys of the DOCA-salt rats and TGRs. The percentage of p16INK4a-positive nuclei in left ventricles of DOCA-salt rats was assessed for all identifiable cells of the myocardium, including myocytes, fibrocytes, endothelial cells, and infiltrating cells, except for the arteries, which were analyzed separately. Analysis of coronary arteries included investigation of all of the arteries seen for the whole section. Evaluation of arteries in both the kidney and heart included all cell types seen in the intima, media, and adventitia and was calculated as a ratio of positive nuclei to total arterial cell nuclei.

### Statistical Analysis

Data were evaluated using the SPSS 14.0 statistical software package (SPSS Inc). Means among different treatment groups were compared using an unpaired manner (Table S1). and Phospho-p38

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### Human Renal Biopsies

Nine kidney biopsies with histopathologic features of hypertensive nephrosclerosis were obtained from 7 male and 2 female patients. Patient age ranged from 48 to 67 years. All of the subjects had a chronic kidney disease stage II to III, and serum creatinine ranged from 1.1 to 3.5 mg/dL.

Slides were derived from diagnostic biopsy samples. In cases where additional slides remained after the completion of all of the diagnostic tests, case subjects were anonymized (only the age of the patient and the pathologist’s diagnosis was supplied). This study was approved by the ethics review board of the University of Erlangen Faculty of Medicine.

### Histopathology of Kidney and Heart

Tissue sections were stained with hematoxylin/eosin, PAS, or Sirius red. In kidneys, the degrees of tubular deterioration (ie, tubular atrophy and dilation), interstitial fibrosis, glomerulosclerosis, and vascular damage were analyzed.17,18 In hearts, we assessed myocardial fibrosis and vascular damage.

### Isolation of Total Kidney RNA and Real-Time PCR

Total RNA was extracted from renal tissue samples. After reverse transcription, quantitative PCR was performed as described previously.19

### Results

**Target Organ Damage in DOCA-Salt Rats**

DOCA-salt–treated animals showed marked increases in tubular deterioration (atrophy and dilation), glomerulosclerosis, interstitial fibrosis, and vascular damage (Table, Figure S1, and Table S2) by 6 weeks. Antihypertensive triple therapy alleviated tubular deterioration, glomerulosclerosis, interstitial fibrosis, and vascular damage, whereas spironolactone had a lesser effect on histopathology, mainly by reducing glomerulosclerosis. In hearts, the myocardial fibrosis score (DOCA 6 weeks versus control: 0.73±0.27 versus 0.25±0.11; P<0.001) and the vascular damage score for intramyocardial arteries (DOCA 6 weeks versus control: 1.05±0.24 versus 0.59±0.18; P<0.01) were significantly elevated after 6 weeks of DOCA treatment.
**Figure 1.** Hypertension induces cell-cycle inhibitor p16\(^{INK4a}\) in DOCA rat kidneys. A, Significant increase in p16\(^{INK4a}\) mRNA levels in kidneys after 6 weeks of DOCA treatment. B, Quantification of p16\(^{INK4a}\) protein expression shows marked increases in the percentage of positively stained nuclei for tubules, glomeruli, interstitium, and arteries after 4 (DOCA 4 weeks) and 6 weeks (DOCA 6 weeks) of hypertension and attenuation under triple (DOCA+TRP) and spironolactone (DOCA+spirono) therapy. Data are shown as means±SEMs. C, Representative p16\(^{INK4a}\) immunoperoxidase stainings of kidneys from control, DOCA 4 weeks, DOCA 6 weeks, DOCA +TRP, and DOCA +spirono rats; magnification, ×200.

**p16\(^{INK4a}\) Expression on mRNA and Protein Level in DOCA-Salt Hypertensive Rat Kidneys**

p16\(^{INK4a}\) mRNA levels significantly increased ≈16-fold after 6 weeks of DOCA administration (Figure 1A). Antihypertensive triple therapy or administration of spironolactone reduced p16\(^{INK4a}\) mRNA levels compared with DOCA 6-week rat kidneys.

Control animals showed only a few p16\(^{INK4a}\)-positively stained tubular, glomerular, interstitial, and arterial nuclei (Figure 1B and 1C). In comparison, the percentage of p16\(^{INK4a}\)-positively stained tubular nuclei was significantly higher in DOCA 4-week kidneys and increased further in DOCA 6-week kidneys. There was also a significant increase in p16\(^{INK4a}\)-positive glomerular, interstitial, and arterial nuclei after 6 weeks of DOCA treatment. Antihypertensive triple therapy significantly reduced the percentage of p16\(^{INK4a}\)-stained nuclei in all 4 of the investigated compartments, whereas spironolactone led to a significant decrease in p16\(^{INK4a}\)-positively stained tubular, glomerular, and arterial but not interstitial nuclei (Figure 1B and 1C).

**Correlation Between Renal p16\(^{INK4a}\) Expression and Histopathology**

p16\(^{INK4a}\) positively stained nuclei were predominantly distributed to injured areas with tubular deterioration and glomerulosclerosis. Nevertheless, p16\(^{INK4a}\)-positive cells were also seen in tubules that did not show tubular damage.

The percentage of tubular p16\(^{INK4a}\)-positive nuclei significantly correlated with the severity of tubular deterioration (correlation with atrophic tubules: \(R=0.6, P<0.001\); with dilated tubules: \(R=0.5, P<0.01\)). Tight correlations were seen between the percentage of glomerular or interstitial p16\(^{INK4a}\)-positive nuclei and the glomerulosclerosis index \((R=0.8; P<0.001)\) or the extent of interstitial fibrosis \((R=0.6; P<0.001;\) please see also Figure S2A to S2D). The vascular damage score correlated with the percentage of p16\(^{INK4a}\)-positive arterial nuclei \((R=0.4; P<0.01)\).

**p-p38 MAPK in DOCA-Salt Hypertensive Rat Kidneys**

Control animals showed few p-p38 positively stained tubular, glomerular, interstitial, and arterial nuclei (Figures 2 and S3). Significant increases in p-p38 expression were found for all 4 of the cell compartments after 6 weeks of DOCA treatment. Antihypertensive triple therapy, as well as spironolactone, significantly reduced the percentage of p-p38–positive nuclei in tubules, glomeruli, interstitium, and arteries (Figure 2). p-p38–positively stained nuclei were predominantly found in damaged areas as described for p16\(^{INK4a}\) (Table S3). Regression analyses showed significant correlations between p-p38 and p16\(^{INK4a}\)-positive nuclei for tubules, glomeruli, interstitium, and arteries in the kidney (Table S3).

**p16\(^{INK4a}\) Expression in Left Ventricles of DOCA-Salt Hypertensive Rats**

In control animals, p16\(^{INK4a}\) protein expression was low in the myocardium and left ventricular arteries (Figures 3 and S4). p16\(^{INK4a}\) protein expression increased significantly after 6 weeks of DOCA administration and was seen in myocytes, fibroblasts, endothelial cells, and other interstitial cells. Re-
gression analyses revealed a correlation between myocardial p16<sup>INK4a</sup> expression and the myocardial fibrosis score (R<sup>2</sup>/H11005 0.5; P/H11005 0.05) but not between arterial p16<sup>INK4a</sup> expression and the vascular damage score (R<sup>2</sup>/H11005 0.06; P/H11005 0.8).

p16<sup>INK4a</sup> Expression in Kidneys of Hypertensive TGRs

Renal p16<sup>INK4a</sup> expression was further investigated in a high (tissue)-renin model for hypertension, the TGR. Nonhypertensive controls showed a low amount of p16<sup>INK4a</sup> expression in tubules, glomeruli, and interstitium (Figures 4 and S5). Hypertensive TGRs displayed significantly higher numbers of p16<sup>INK4a</sup> positively stained nuclei for all 3 of the investigated compartments. Administration of AT<sub>1</sub> receptor blocker losartan resulted in a highly significant decrease in p16<sup>INK4a</sup> expression for tubular, glomerular, and interstitial cell nuclei.

p16<sup>INK4a</sup> Expression in Renal Biopsies With Hypertensive Nephropathy

Quantification of p16<sup>INK4a</sup> expression was performed in biopsies from 9 patients with hypertensive nephropathy. Based on the regression with age in previously published normal kidney specimens, we calculated an expected p16<sup>INK4a</sup> expression value for every biopsy sample. We found the measured p16<sup>INK4a</sup> expression in biopsies with hypertensive changes to be significantly higher in tubules, glomeruli, and interstitium when compared with that expected for kidney age (Figures 5 and S6).

Figure 2. Effects of hypertension and therapy on p-p38 MAPK in DOCA-salt rat kidneys. p-p38 expression is predominantly found after 6 weeks of uncontrolled hypertension (DOCA 6 weeks) and is attenuated by triple (DOCA + TRP) and spironolactone (DOCA + spirono) therapy. The percentage of p-p38–positive nuclei is illustrated for tubular (A), glomerular (B), interstitial (C), and arterial (D) cells. Data are shown as means±SEMs.

Figure 3. Hypertension induces p16<sup>INK4a</sup> expression in left ventricles of DOCA-salt rats. Quantification of p16<sup>INK4a</sup> protein expression within the myocardium (A) and left ventricular arteries (B) shows a significant increase in the percentage of positively stained nuclei after 6 weeks of uncontrolled hypertension (DOCA 6 weeks). Data are shown as means±SEMs.
Discussion

This study provides evidence that hypertension induces senescence in rodents and humans. p16<sup>INK4a</sup> expression significantly correlated with the histopathologic changes seen in hypertensively damaged kidney and myocardium. Renal aging and several kidney diseases are characterized by tubular atrophy and interstitial fibrosis that correlate with p16<sup>INK4a</sup> expression, suggesting that this phenotype is a common end point for senescence-induced histopathologic changes. Similarly, hypertensive heart disease and the aging heart both show left ventricular hypertrophy, fibrosis, and impairments in ventricular performance and coronary hemodynamics. Thus, the hypertension-induced increases in p16<sup>INK4a</sup> expression in the left ventricular myocardium and arteries could also result in a widespread irreversible cell cycle arrest with accumulation of senescent cells. We found increases in p16<sup>INK4a</sup> through systemic hypertension in 2 different tissues (kidney and heart) that are target organs for hypertensive damage. However, it seems that this result cannot be conferred to pulmonary hypertension, because Yu et al<sup>21</sup> did not detect changes in pulmonary p16<sup>INK4a</sup> expression in mice with hypoxia-induced pulmonary hypertension.

In renal biopsies from patients with hypertensive nephrosclerosis and impaired renal function, high BP strongly induced p16<sup>INK4a</sup> expression in tubules, glomeruli, and interstitium. Thus, the increase in p16<sup>INK4a</sup> expression beyond normal aging reflects the added burden of hypertension and confirms that senescence induced by high BP is not restricted to hypertensive rat models but can be extended to human subjects.

p16<sup>INK4a</sup> induction by hypertension in the DOCA model is due to high BP and activation of the mineralocorticoid receptor by DOCA. Antihypertensive triple medication reduced BP, prevented induction of cellular senescence, and coincided with a significant amelioration of renal histopathology. These effects occurred without any interference with the mineralocorticoid receptor and thereby are consistent with the idea that BP itself causes an induction of senescence. Our data also point to direct effects of angiotensin II and aldosterone on cellular senescence beyond the effects of hypertension, per se. Spironolactone, administered in a nonantihypertensive dose, improved renal histopathology and reduced p16<sup>INK4a</sup>. Mineralocorticoids have proinflammatory effects, induce oxidative stress, and enhance proliferation. All of these factors may contribute to cellular senescence. In the angiotensin II–dependent TGR model, losartan lowered p16<sup>INK4a</sup> despite a very minor effect on BP. Two recent articles demonstrated direct effects of angiotensin II on cellular senescence in vascular smooth muscle cells<sup>23,24</sup>; one of those articles showed evidence that the increases seen in p16<sup>INK4a</sup> expression are at least partially mediated by aldosterone. Wolf et al reported that angiotensin-converting enzyme inhibition reduced the glomerular expression of p16<sup>INK4a</sup>. Together with these previous reports, our data support the notion that aldosterone and angiotensin II contribute to renal cellular senescence via hemodynamic and nonhemodynamic effects. Because overall the changes seen with BP-lowering triple therapy were greater than with spironolactone, hemodynamic effects seem to be more important for the induction of cellular senescence.

p38 MAPK activation likely contributes to the induction of senescence-associated cell cycle inhibitor p16<sup>INK4a</sup>. p38 MAPK has been identified as a positive upstream regulator of p16<sup>INK4a</sup> and plays an important role in cellular senescence of different origins.<sup>26,27</sup> The 4 p38 MAPK isoforms (p38<sub>α</sub>, p38<sub>β</sub>, p38<sub>γ</sub>, and p38<sub>δ</sub>) are activated by a variety of cellular stresses. Aldosterone activates p38 MAPK in vascular smooth muscle cells.<sup>25,28</sup> Protective effects of p38 MAPK inhibition against hypertensive target organ damage were
shown in different hypertensive animal models. Our correlation analyses show a significant relationship between p16INK4a and p-p38 expression in tubules, glomeruli, interstitium, and arteries and suggest that p38 MAPK activation induces p16INK4a. Given these data, p38 MAPK activation might be even more important in forms of hypertension with an activated renin-angiotensin-aldosterone system. Future experiments should also investigate the sequential activation of the p38 MAPK-p16INK4a signaling pathway, e.g., by hypertension stress signals like angiotensin II in isolated cells.

**Perspectives**

Our data demonstrate that hypertension causes the induction of senescence-associated cell cycle inhibitor p16INK4a in the kidney and heart. Somatic cellular senescence could provide a common pathway by which age, disease, and injury exhaust the reserve of somatic cells that are capable of cell division and thereby cell renewal, cellular skills indispensable for organ repair and integrity. The resulting irreversible cell-cycle arrest of organ-specific cells contributes to a disturbed organ homeostasis with a reduced regenerative capacity that ultimately leads to hypertensive target organ damage and to increased susceptibility to other injury- and disease-induced damage. Strategies to prevent hypertension-induced senescence consist of the following: (1) lowering BP; (2) renin-angiotensin-aldosterone system blockade; and (3) inhibition of the p38 MAPK-p16INK4a signaling pathway. For the first task, several potent drug classes are available. Indeed, one might prefer drugs also fulfilling the second task. In addition to the widely used angiotensin-converting enzyme inhibitors and AT1 receptor blockers, mineralocorticoid-receptor blockers such as spironolactone or eplerenone may gain importance. New drug classes like renin inhibitors or aldosterone synthesis blockers may become of importance in the future. Finally, the last target for intervention could be the signaling pathways involved in somatic cellular senescence. However, because such substances are not yet identified, this task has to be tackled in an experimental setting and, therefore, has no instant consequences on current drug regimens.

**Acknowledgment**

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**Disclosures**

None.

**References**


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HYPERTENSION INDUCES SOMATIC CELLULAR SENESCENCE IN RAT AND HUMAN BY INDUCTION OF CELL CYCLE INHIBITOR P16^{INK4A}

Jens H. Westhoff\textsuperscript{1}, Karl F. Hilgers\textsuperscript{2}, Mario P. Steinbach\textsuperscript{1}, Andrea Hartner\textsuperscript{2}, Bernd Klanke\textsuperscript{2}, Kerstin Amann\textsuperscript{3}, Anette Melk\textsuperscript{1}

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Short title: Hypertension induces somatic cellular senescence

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Expanded Methods, Supplemental Figures & Tables

Animal models of hypertension

All procedures performed on animals were done in accordance with institutional guidelines for animal research and were approved by the local government authorities.

DOCA-salt hypertension

For DOCA-salt studies an experimental protocol was used that has been published previously.\textsuperscript{1} Briefly, rats were housed in a room maintained at 22 ± 2°C, exposed to a 12-hour dark/light cycle. 3 months old male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) at an average weight of 150 g underwent left unilateral nephrectomy. After 2 weeks of recovery, 21 day-release DOCA pellets containing 50 mg of DOCA (Innovative Research of America, Sarasota, FL) were implanted subcutaneously by incision of the right flank under ether anesthesia. The animals received a second pellet 21 days after the first pellet. Control animals were unilaterally nephrectomized, but did not receive DOCA pellets (Control). All animals were allowed unlimited access to chow (no.1320; Altromin, Lage, Germany) and isotonic saline (10 g sodium chloride/L) starting with the first day of DOCA treatment. A subgroup of DOCA rats received additional medication for the last two weeks (after 4 weeks of hypertension), consisting of either the aldosterone receptor antagonist spironolactone (DOCA+spirono; 100 mg/kg/d, dissolved in sesame oil by daily gavage) or an antihypertensive triple therapy combining thiazide-type diuretic hydrochlorothiazide, direct vasodilator hydralazine and peripheral sympatholytic agent reserpine (DOCA+TRP) in the drinking water. Animals were sacrificed after 4 weeks (DOCA 4 wk, n=6) and 6 weeks (Controls, n=8; DOCA 6 wk, n=14; DOCA+spirono, n=7; DOCA+TRP, n=8). Noteworthy, six of the 14 DOCA 6 wk rats received daily gavage of sesame oil and, as they did not differ from DOCA 6 wk rats, were summarized within the same group.

Urine was collected for 24 h before sacrifice for measurement of proteinuria (Bio-Rad Protein Assay; Bio-Rad Laboratories, Munich, Germany). At the day of sacrifice, animals were
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equipped with a femoral catheter under enflurane anesthesia, and intra-arterial BP was measured in conscious rats 4 h after anesthesia.

After sacrifice, the right kidney and the left ventricle were excised. Before weighing, adjacent tissue was carefully removed and kidneys were decapsulated. Part of each kidney was immediately snap frozen in liquid nitrogen for later RNA extraction; a second part was fixed in 3% paraformaldehyde and paraffin-embedded. 2 µm sections were cut with a Leica RM 2165 microtome (Leica Instruments, Nussloch, Germany) for PAS and H&E stainings and 3 µm sections were cut for immunostainings. Left ventricles were paraformaldehyde fixed, paraffin-embedded and cut as described for the kidneys.

**TGR hypertensive rats**

Transgenic rats heterozygous for the mouse Ren-2 gene are regarded as a high (tissue-) renin model with angiotensin II dependent hypertension, although plasma renin is low. For this study, part of an experimental protocol was used that has been published previously. Twelve 12 week old male TGR rats and age-matched Sprague-Dawley-Hannover (SD; n=7) controls (Möllegaard, Eijby, Denmark) were housed as described for DOCA-salt rats above. Five of the TGR rats received AT1 receptor blocker losartan (1 mg/kg body weight per day) intraperitoneally by osmotic minipumps (Alzet model 2004; Alza Scientific Products, Palo Alto, CA, USA) for 4 weeks. At the day of sacrifice, all animals were equipped with femoral catheters for intra-arterial BP monitoring in conscious rats 4 h after anesthesia. Kidneys were harvested as described for DOCA-salt rats. 3 µm sections were cut for PAS and H&E as well as for immunostainings.

**Human renal biopsies**

Nine kidney biopsies with histopathological features of hypertensive nephrosclerosis were obtained from seven male and two female patients. Patient age ranged from 48 to 67 yr (mean
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± SD: 58.8 ± 7.4 yr). All subjects had a CKD stage II-III, serum creatinine ranged from 1.1 mg/dl to 3.5 mg/dl (mean ± SD: 1.88 ± 0.64 mg/dl).

Human kidney slides were obtained from diagnostic biopsy samples. In cases were additional slides remained after completion of all diagnostic tests, cases were anonymized (only the age of the patient and the pathologist's diagnosis was supplied). This study was approved by the ethics review board of the University of Erlangen's Faculty of Medicine.

**Histopathology of kidney and heart**

Tissue sections were stained with H&E or PAS, respectively. Photographs of ten random high power field pictures (HPFs; 200x magnification; Leica DFC 320) were taken of each rat kidney cortex using a Leica DM LB2 digitizing microscope and subsequent analysis was performed using QWin V3 software package.

Diameter of all proximal and distal as well as atrophic or dilated tubules was measured. Number of nuclei per tubular cross section as well as thickness of tubular basement membrane was assessed for proximal, distal, atrophic and dilated tubules in the same pictures. In addition, tubular basement membrane was assigned to as either smooth or wrinkled. Criteria for specification as “atrophic tubule” were at least the simultaneous appearance of reduced tubular diameter and thickened tubular basement membrane. Tubules were specified as “dilated” when tubular diameter was increased and tubular epithelium was flattened. Tubular deterioration in the following comprises both atrophic and dilated tubules.

Glomerulosclerosis was evaluated in kidney sections stained with PAS reagent as described before. A score of 0 to 4 was used to quantify glomerulosclerosis as follows: 0, normal glomerulus; 1, mesangial expansion or sclerosis involving 25% of the glomerular tuft; 2, sclerosis 25% to 50%; 3, sclerosis 50% to 75%, segmental extracapillary fibrosis or proliferation, or both; 4, global sclerosis (>75%) or global extracapillary fibrosis or proliferation, or complete collapse of the glomerular tuft.
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The degree of renal and myocardial fibrosis was measured using Sirius Red stainings and a semiquantitative scoring system. Fibrosis in kidneys and hearts was evaluated by analysis of 20 HPF’s (100x magnification) and subsequent application of the following score for each HPF: 0, no fibrosis; 1, 0-25% fibrosis; 2, 25-50% fibrosis; 3, 50-75% fibrosis; 4, >75% fibrosis. The vascular damage score was analyzed using a semiquantitative scoring system ranging from 0 to 4 to quantify vascular damage as described before. Then the mean score per animal was calculated and the mean per group was obtained as the mean of all animal scores.

**Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was extracted from renal tissue samples according to the single step method of Chomczynski using TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA). Purity and yield was determined photometrically. Transcription of 1 µg total RNA into cDNA was done using MMLV reverse transcriptase and random primers (Invitrogen, Karlsruhe, Germany). Quantitative PCR was performed as described previously. The mRNA expression of p16 in control kidneys was set as 1. Groups for DOCA-salt kidneys consisted of control (n=7), DOCA 4 wk (n=3), DOCA 6 wk (n=9), DOCA+TRP (n=7) and DOCA+spirono (n=7) samples.

**Immunohistochemistry for p16**

Immunoperoxidase staining for p16 was performed using paraffin-embedded tissue as described previously. The same protocol was used for kidney sections of DOCA-salt and TGR rats as well as for human renal biopsies and left ventricular cross-sections of DOCA-salt rats. Briefly, sections were deparaffinized and hydrated. The sections were immersed at 3% H₂O₂ methanol to inactivate endogenous peroxidase. Slides were blocked with 20% normal goat serum over night. Tissue sections were then incubated for 1 h at room temperature with the primary antibody (mouse monoclonal antibody, Clone F-12, Santa Cruz Biotechnologies, Heidelberg, Germany) and rinsed with PBS. Following 30 min of incubation with the Envision monoclonal system (Dako, Hamburg, Germany) sections were washed again in PBS.
Visualization was performed using the diaminobenzidine (DAB) substrate kit (Dako, Hamburg, Germany). The slides were counterstained with hematoxylin and mounted. Analysis was done by counting 10 HPFs (200x magnification) by a blinded observer. For kidney sections of DOCA-salt studies (Control, n=7; DOCA 4 wk, n=6; DOCA 6 wk, n=14; DOCA+TRP, n=8; DOCA+spirono, n=7) and TGR studies (SD, n=7; TGR, n=7; TGR+losartan, n=5) percentage of positive nuclei in comparison to total nuclei number was assessed separately for tubules, glomeruli, interstitium and renal arteries. In contrast, percentage of p16\textsuperscript{INK4a} positive nuclei in left ventricles of DOCA-salt rats (Control, n=7; DOCA 4 wk, n=6, DOCA 6 wk, n=4) was assessed for all identifiable cells of the myocardium – including myocytes, fibrocytes, endothelial cells and infiltrating cells - except for the arteries, which were analyzed separately. Analysis of coronary arteries included investigation of all arteries seen for the whole cross-section. Evaluation of arteries in both kidney and heart included all cell types seen in intima, media and adventitia and was calculated as ratio of positive nuclei to total arterial cell nuclei.

**Immunohistochemistry for p-p38**

Immunoperoxidase staining for p-p38 was done using paraffin embedded tissue. 3 μm sections were deparaffinized and rehydrated. Heat-induced antigen retrieval was performed using microwave treatment in 10mM sodium citrate buffer (pH 6.0) for 20 min. Subsequently, sections were immersed in 3% H\textsubscript{2}O\textsubscript{2} methanol to inactivate endogenous peroxidase. Following, slides were blocked with 20% normal goat serum for 10 min. Tissue sections were then incubated for 1 h at room temperature with the primary antibody (Phospho-p38 MAP Kinase (Thr180/Tyr182) (12F8) Rabbit Monoclonal Antibody, Cell Signaling Technology, Inc., Beverly, MA, USA) and rinsed with PBS. Following 30 min of incubation with the Envision monoclonal system (Dako, Hamburg, Germany) sections were washed again in PBS. Visualization was performed using the DAB substrate kit (Dako, Hamburg, Germany). The slides were counterstained with hematoxylin and mounted. Analysis was performed for kidney sections of DOCA-salt rats as described under p16\textsuperscript{INK4a} immunohistochemistry.
Statistical analysis

Data was evaluated using the SPSS 14.0 statistical software package (SPSS Inc., Chicago, IL, USA). Means among different treatment groups were compared using analysis of variance (ANOVA), and *t*-tests with Bonferroni correction were applied for multiple pair-wise comparisons. Correlation analyses were performed by two-sided bivariate regressions. For the human biopsies, the expected $p16^{\text{INK4a}}$ expression was calculated using the published regression formulas:\(^\text{10}\): tubular $p16^{\text{INK4a}} = 0.43 \times \text{age} - 0.47$; glomerular $p16^{\text{INK4a}} = 0.22 \times \text{age} + 5.31$; interstitial $p16^{\text{INK4a}} = 0.18 \times \text{age} - 1.25$. Expected and measured $p16^{\text{INK4a}}$ expression was compared using unpaired *t*-test.
References


Table S1. Blood pressure and proteinuria in deoxycorticosterone acetate (DOCA)-salt rats. All values are given as mean ± SD.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>DOCA 4 wk</th>
<th>DOCA 6 wk</th>
<th>DOCA + TRP</th>
<th>DOCA + spirono</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure (mmHg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>115 ± 4</td>
<td>158 ± 7*</td>
<td>168 ± 8†</td>
<td>125 ± 7‡</td>
<td>159 ± 7</td>
</tr>
<tr>
<td>Proteinuria (mg/d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>28 ± 4</td>
<td>194 ± 110</td>
<td>483 ± 67§</td>
<td>99 ± 20§</td>
<td>376 ± 69</td>
</tr>
</tbody>
</table>

* p<0.01 vs. control of same group.
† p<0.001 vs. control of same group.
‡ p<0.001 vs. DOCA 6 wk of same group.
§ p=0.001 vs. control of same group.
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Table S2. Extended table on renal tubular histopathology in DOCA-salt hypertensive rats and influence of antihypertensive triple (DOCA+TRP) and non-antihypertensive spironolactone (DOCA+spirono) therapy. All values are given as mean ± SD. In comparison to controls, DOCA 6 wk rats showed decreased proximal tubular diameters. TBM thickness, cell number per TCS and percentage of tubules with wrinkled TBM’s were significantly higher in DOCA 6 wk rats. Triple therapy normalized proximal tubular diameter and reduced percentage of wrinkled TBM’s. Spironolactone decreased the number of dilated tubules. Proximal tubular diameter under triple therapy was comparable to the control group. No significant effect on TBM thickness and mean cell number per TCS was seen under any of both therapies.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>DOCA 4 wk</th>
<th>DOCA 6 wk</th>
<th>DOCA + TRP</th>
<th>DOCA + spirono</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diameter [µm]</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proximal tubules</td>
<td>46.1±1.1</td>
<td>44.8±4.9</td>
<td>40.8±2.7*</td>
<td>47.0±1.8†</td>
<td>44.0±3.0</td>
</tr>
<tr>
<td>Distal tubules</td>
<td>32.4±2.0</td>
<td>36.0±3.2</td>
<td>35.2±5.5</td>
<td>34.2±5.0</td>
<td>35.1±5.1</td>
</tr>
<tr>
<td>atrophic tubules</td>
<td>None</td>
<td>27.6±5.9†</td>
<td>27.8±5.2‡</td>
<td>24.6±7.2‡</td>
<td>27.5±3.5‡</td>
</tr>
<tr>
<td>dilated tubules</td>
<td>None</td>
<td>48.0±4.9</td>
<td>53.5±10.4 §</td>
<td>53.5±3.0 §</td>
<td>46.5±9.2</td>
</tr>
<tr>
<td><strong>Thickness of TBM [µm]</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proximal tubules</td>
<td>0.97±0.10</td>
<td>1.10±0.17</td>
<td>1.38±0.18*</td>
<td>1.19±0.30</td>
<td>1.18±0.14</td>
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<tr>
<td>Distal tubules</td>
<td>0.90±0.14</td>
<td>1.09±0.15</td>
<td>0.98±0.16</td>
<td>1.06±0.25</td>
<td>1.10±0.19</td>
</tr>
<tr>
<td>atrophic tubules</td>
<td>None</td>
<td>1.34±0.21</td>
<td>1.62±0.32‖§</td>
<td>1.74±0.17‖§</td>
<td>1.67±0.64‖§</td>
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<tr>
<td>dilated tubules</td>
<td>None</td>
<td>1.17±0.04</td>
<td>1.31±0.19†,¶</td>
<td>1.28±0.71</td>
<td>1.21±0.20</td>
</tr>
<tr>
<td><strong>Nuclei per TCS (#)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proximal tubules</td>
<td>4.23±0.41</td>
<td>5.65±1.44‖</td>
<td>6.79±0.51*</td>
<td>5.90±0.91*</td>
<td>6.46±0.69*</td>
</tr>
<tr>
<td>Distal tubules</td>
<td>5.89±1.17</td>
<td>6.25±0.80</td>
<td>7.01±1.32</td>
<td>5.70±0.87</td>
<td>6.38±0.49</td>
</tr>
<tr>
<td>atrophic tubules</td>
<td>None</td>
<td>5.51±2.24</td>
<td>6.24±1.14**</td>
<td>6.11±2.26</td>
<td>6.49±0.86**</td>
</tr>
<tr>
<td>Distal tubules</td>
<td>None</td>
<td>5.88±0.88</td>
<td>8.72±2.75**</td>
<td>7.50±0.71</td>
<td>9.05±3.64**</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Ratio wrinkled TBMs (%)</th>
<th>0.0±0.0</th>
<th>23.9±28.0</th>
<th>51.2±22.3†</th>
<th>15.3±19.4‡</th>
<th>31.4±19.4#</th>
</tr>
</thead>
</table>

TBM, tubular basement membrane; TCS, tubular cross section.

* p<0.01 vs. control of same group
† p<0.01 vs. DOCA 6 wk of same group
‡ p<0.01 vs. diameter of proximal tubules of control
§ p<0.05 vs. diameter of distal tubules of control
¶ p<0.01 vs. TBM thickness of proximal tubules of control
* p<0.01 vs. TBM thickness of distal tubules of control
# p<0.05 vs. control of same group
** p<0.05 vs. # nuclei per TCS of proximal tubules of control
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Table S3. Regression analyses for p-p38 and p16\textsuperscript{INK4a} expression in the kidney of DOCA-salt hypertensive rats.

<table>
<thead>
<tr>
<th></th>
<th>Pearson coefficient (R)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-p38 vs. p16\textsuperscript{INK4a}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tubular</td>
<td>0.661</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>glomerular</td>
<td>0.582</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>interstitial</td>
<td>0.552</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>renal arterial</td>
<td>0.603</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Online Figure Legends

**Figure S1. Histopathology of hypertensive nephropathy and influence of antihypertensive therapy and mineralocorticoid-receptor blockade in DOCA-salt rats.** Representative PAS stainings show typical histological alterations of tubular atrophy and dilation, glomerulosclerosis and interstitial fibrosis that are seen under DOCA treatment for either 4 (DOCA 4 wk) or 6 weeks (DOCA 6 wk). Antihypertensive triple therapy (DOCA+TRP) and non-antihypertensive administration of spironolactone (DOCA+spirono) attenuate the damage. Controls did not receive DOCA treatment. Magnification, x200.

**Figure S2. P16\(^{\text{INK4a}}\) expression correlates with histopathological features of hypertension in DOCA-salt rat kidneys.** Regression analyses were performed comparing percentage of atrophic (A) and dilated (B) tubules, glomerulosclerosis (GS) index (C) and interstitial fibrosis (IF) index (D) with percentage of p16\(^{\text{INK4a}}\) positive nuclei in the particular compartment for each animal. Different treatment groups are illustrated as (♦) for controls, (□) for DOCA 4 wk, (■) for DOCA 6 wk, (●) for DOCA+TRP and (○) for DOCA+spirono.

**Figure S3. Effects of hypertension and therapy on p-p38 MAPK in DOCA-salt rat kidneys.** Representative p-p38 immunoperoxidase stainings of kidneys from control, DOCA 4 wk, DOCA 6 wk, DOCA+TRP and DOCA+spirono rats; magnification, x200. P-p38 expression significantly increases after 6 weeks of uncontrolled hypertension (DOCA 6 wk) and is attenuated by triple (DOCA+TRP) and spironolactone (DOCA+spirono) therapy.

**Figure S4. Hypertension induces p16\(^{\text{INK4a}}\) expression in left ventricles of DOCA-salt rats.** Representative p16\(^{\text{INK4a}}\) immunoperoxidase stainings of left ventricles (A) and of left ventricular arteries (B) for control, DOCA 4 wk and DOCA 6 wk rats; magnification, x400.
Figure S5. Hypertension induces senescence-associated cell cycle inhibitor p16\textsubscript{INK4a} in TGR hypertensive rat kidneys. P16\textsubscript{INK4a} protein expression was assessed for tubular, glomerular and interstitial cells in Sprague-Dawley control rats (SD), Ren-2 transgenic rats (TGR), and TGR rats receiving AT\textsubscript{1} receptor blocker losartan for 4 weeks (TGR+losartan). Representative p16\textsubscript{INK4a} immunoperoxidase stainings showing increases in positively stained nuclei in TGR rat kidneys that are attenuated by additional AT\textsubscript{1} receptor blocker administration; magnification, x200.

Figure S6. P16\textsubscript{INK4a} expression in kidney biopsies showing hypertensive nephrosclerosis. P16\textsubscript{INK4a} protein expression of kidney biopsy specimens from hypertensive patients is shown for tubules, glomeruli and interstitium; magnification, x400.
Figure S1
Figure S2

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A

Tubular p16\textsuperscript{INK4a} positive nuclei (%) vs. Atrophic tubules (%)

\[ y = 77.24x + 19.54 \]
\[ R = 0.5895 \]
\[ p < 0.001 \]

B

Tubular p16\textsuperscript{INK4a} positive nuclei (%) vs. Dilated tubules (%)

\[ y = 70.43x + 22.78 \]
\[ R = 0.4678 \]
\[ P < 0.01 \]

C

Glomerular p16\textsuperscript{INK4a} positive nuclei (%) vs. GS index

\[ y = 11.77x - 4.80 \]
\[ R = 0.7915 \]
\[ P < 0.001 \]

D

Interstitial p16\textsuperscript{INK4a} positive nuclei (%) vs. Fibrosis index

\[ y = 6.67x + 5.80 \]
\[ R = 0.5528 \]
\[ p < 0.001 \]
Figure S3
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Figure S4
Figure S6

Hypertension induces somatic cellular senescence