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

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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{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.

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The experimental protocol has been published elsewhere. At the angiotensin II type 1 (AT1) receptor blocker losartan for 4 weeks.

Transgenic (mRen2)27 hypertensive rats (TGRs) heterozygous for the mouse Ren-2 gene are regarded as a high (tissue-) renin model with angiotensin II–dependent hypertension, although plasma angiotensin is low. Twelve-week–old male TGRs (n = 12) and Sprague-Dawley controls (n = 7) were used. Five of the TGRs received angiotensin II type 1 (AT1) receptor blocker losartan for 4 weeks. The experimental protocol has been published elsewhere.

Statistical Analysis
Data were evaluated using the SPSS 14.0 statistical software package (SPSS Inc). Means among different treatment groups were compared using ANOVA, and t tests with Bonferroni correction were applied for multiple pairwise comparisons. Correlation analyses were performed by 2-sided bivariate regressions. For the human biopsies, the expected p16INK4a expression was calculated using the published regression formulas. Expected and measured p16INK4a expression were compared using an unpaired t test.

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.

Deoxycorticosterone Acetate-Salt Hypertension
The experimental protocol used for deoxycorticosterone acetate (DOCA)-salt studies has been published previously. In addition, 2 treatment groups were investigated: antihypertensive triple therapy (hydrochlorothiazide, hydralazine, and reserpine [DOCA+TRP]) and spironolactone in a nonantihypertensive dose (DOCA+spirono).

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. 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.

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

p16\(^{INK4a}\) mRNA levels significantly increased \(\approx 16\)-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\(^{INK4a}\)-positive 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 administration. 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\(^{INK4a}\) (Figure 1A). 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 p16INK4a expression and the myocardial fibrosis score (R = 0.5; P < 0.05) but not between arterial p16INK4a expression and the vascular damage score (R = 0.06; P = 0.8).

p16INK4a Expression in Kidneys of Hypertensive TGRs

Renal p16INK4a expression was further investigated in a high (tissue)-renin model for hypertension, the TGR. Nonhypertensive controls showed a low amount of p16INK4a expression in tubules, glomeruli, and interstitium (Figures 4 and S5). Hypertensive TGRs displayed significantly higher numbers of p16INK4a positively stained nuclei for all 3 of the investigated compartments. Administration of AT1 receptor blocker losartan resulted in a highly significant decrease in p16INK4a expression for tubular, glomerular, and interstitial cell nuclei.

p16INK4a Expression in Renal Biopsies With Hypertensive Nephropathy

Quantification of p16INK4a 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 p16INK4a expression value for every biopsy sample. We found the measured p16INK4a 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).
Thus, the hypertension-induced increases in p16INK4a 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 p16INK4a 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.\(^{21}\) did not detect changes in pulmonary p16INK4a expression in mice with hypoxia-induced pulmonary hypertension.

In renal biopsies from patients with hypertension nephrosclerosis and impaired renal function, high BP strongly induced p16INK4a expression in tubules, glomeruli, and interstitium. Thus, the increase in p16INK4a 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.

p16INK4a 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, impaired renal histopathology and reduced p16INK4a. Mineralocorticoids have proinflammatory effects, induce oxidative stress, and enhance proliferation.\(^{22}\) All of these factors may contribute to cellular senescence. In the angiotensin II–dependent TGR model, losartan lowered p16INK4a despite a very minor effect on BP. Two recent articles demonstrated direct effects of angiotensin II on cellular senescence in vascular smooth muscle cells,\(^{23,24}\) one of those articles showed evidence that the increases seen in p16INK4a expression are at least partially mediated by aldosterone. Wolf et al. reported that angiotensin-converting enzyme inhibition reduced the glomerular expression of p16INK4a.\(^{25}\) 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 p16INK4a. p38 MAPK has been identified as a positive upstream regulator of p16INK4a and plays an important role in cellular senescence of different origins.\(^{26,27}\) The 4 p38 MAPK isoforms (p38α, p38β, p38γ, and p38δ) are activated by a variety of cellular stresses. Aldosterone activates p38 MAPK in vascular smooth muscle cells.\(^{25,28}\) 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, interstitial, 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, eg, by hypertensive 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 p16INK4a signaling pathways. 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.

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Disclosures
None.

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