Growth Arrest Specific Protein 6 Participates in DOCA-Induced Target-Organ Damage

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Abstract—Growth arrest–specific protein 6 (Gas 6) is involved in inflammatory kidney diseases, vascular remodeling, cell adhesion, and thrombus formation. We explored a role for Gas 6 in aldosterone-induced target organ damage. We observed that Gas 6 was upregulated in rats with high aldosterone levels. Mineralocorticoid receptor blockade prevented target organ damage and decreased the elevated Gas 6 expression. Vascular smooth muscle cells given aldosterone increased their Gas 6 expression in vitro. To test the pathophysiological relevance, we investigated the effects of deoxycorticosterone acetate (DOCA) on Gas 6 gene-deleted (−/−) mice. After 6 weeks DOCA, Gas 6−/− mice developed similar telemetric blood pressure elevations compared to wild-type mice but were protected from cardiac hypertrophy. Cardiac expression of interleukin 6 and collagen IV was blunted in Gas 6−/− mice, indicating reduced inflammation and fibrosis. Gas 6−/− mice also had an improved renal function with reduced albuminuria, compared to wild-type mice. Renal fibrosis and fibronectin deposition in the kidney were also reduced. Gas 6 deficiency reduces the detrimental effects of aldosterone on cardiac and renal remodeling independent of blood pressure reduction. Gas 6 appears to play a role in mineralocorticoid receptor-mediated target organ damage. Furthermore, because warfarin interferes with Gas 6 protein expression, the findings could be of clinical relevance for anticoagulant choices. (Hypertension. 2009;54:00-00.)

Key Words: Gas 6 ■ aldosterone ■ cardiac hypertrophy ■ albuminuria ■ inflammation

The renin-angiotensin-aldosterone system (RAAS) mediates cardiovascular and renal inflammation and fibrosis. RAAS activation can cause hypertension, atherosclerosis, and cardiac and renal failure, which can be prevented with blocking angiotensin (Ang) II action.1,2 Ang II is a potent direct stimulus of aldosterone (Ald) synthesis; however, Ald can affect cardiovascular and renal diseases independent of Ang II.3,4 Ald signaling is mediated via the mineralocorticoid receptor (MR), and MR blockade reduces cardiovascular and renal morbidity.5,6 Nonetheless, MR-regulated pathways are only incompletely understood. Growth arrest-specific protein 6 (Gas 6) is involved in vascular remodeling, cell adhesion, and thrombus formation, all of which influence cardiovascular outcomes.7,8 Manfioletti et al first described Gas 6 as a protein in NIH3T3 fibroblasts with highly increased expression during G0 phase of the cell cycle.9 Gas 6 can be detected in plasma and in numerous tissues, including heart, kidney, and adrenal gland.10,11 The Gas 6 gene lies on human chromosome 13q34.12 Gas 6 is a vitamin K–dependent protein and interacts with phosphatidylycerine motifs via a Gla-sequence. In addition, the Gas 6 carboxyterminal domains can bind to the receptor tyrosine kinases Axl, Sky, and Mer. Melaragno et al described Gas 6 and Axl protein upregulation after experimental vascular injury.13 Ang II can induce protein expression of both Gas 6 and Axl in vascular smooth muscle cells (VSMCs); however, the effects of aldosterone on Gas 6 remain unknown.13,14 We observed Gas 6 expression in a RAAS-dependent rat model with high Ald levels and excellent response to MR blockade. The findings caused us to investigate relationships between Gas 6 and MR signaling in greater detail.

Methods

Animals

We adhered to institutional experimental animal guidelines corresponding to those of the American Physiological Society. We used double transgenic rats (dTGR) harboring human renin and human angiotensinogen genes as described elsewhere.15 Briefly, dTGR were treated with the MR blocker spironolactone (20 mg/kg/d) from week 4 to 7 of age; age-matched untreated dTGR and Sprague–Dawley rats served as controls. Gas 6−/− mice on an FVB background were generated as published.16 We treated the mice with DOCA to supply the animals with a defined additional dose of mineralocorticoids, as described by Hartner et al.17 Mice (16 to 18 g, averaging 6 weeks of age) were assigned randomly to the respective treatment groups. DOCA (400 mg/kg/d) or saline (as control) were injected subcutaneously. Gas 6−/− mice served as controls. The animals were killed at 8 weeks after DOCA treatment. Hearts and kidneys were removed and blood pressure was measured telemetrically. The left atria of the hearts were harvested, and whole kidneys were excised. Hearts and kidneys were weighed, snap frozen in liquid nitrogen, and stored at −80°C until further analysis.

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age) were uninephrectomized. Two weeks later, a 21-day-release DOCA pellet (50 mg, Innovative Research of America) was implanted subcutaneously. Control animals were sham operated. After 3 weeks the pellet was replaced. Blood pressure, heart rate, and locomotion were monitored by radiotelemetry, using a pressure-sensing catheter (TA11PA-C20) positioned through the right femoral artery into the abdominal aorta. The transmitter was placed in a subcutaneous pocket along the right flank.\textsuperscript{18} Recordings began 7 days after surgery, when the mice had regained circadian rhythm and surgery-related hemodynamic changes had abated. We collected data continuously every 5 minutes for 10 seconds using Dataquest software (A.R.T. 2.1, Data Science International). Echocardiography was performed in at least 5 animals of each group in anesthetized mice (2% Isoflurane) placed on a heated platform. We assessed cardiac morphology and function with a high-resolution (40 MHz) transducer using VisualSonics Vevo 770 High-Resolution Imaging System. Albumin excretion in urine was measured by ELISA (CellTrend) and normalized to urine creatinine (modified Jaffe reagent, Beckman Instruments). For immunohistochemistry, organs were washed with ice-cold saline and frozen via isopentane (−35°C) at −80°C. Histopathologic analysis was carried out on 4% paraformaldehyde-fixed paraffin sections (2-μm thickness), cut on a rotation microtome (Leica RM2245), stained with trichrome after Mas-son–Goldner, and examined by light microscopy using a Zeiss Axioplan microscope. For the semiquantitative analysis of renal histopathology we used a grading scale in which 0 indicated no damage, 1 indicated very few, 2 a few, 3 moderate, 4 pronounced, and 5 very severe damage. We analyzed 15 areas per section in each of 5 mice from each group. Immunostaining was present, 1 indicated very weak, 2 weak, 3 moderate, 4 pronounced, and 5 very strong staining signal. For each marker 3 experiments were examined at least 5 different slights from 5 animals per group were analyzed (400×), results are stated in arbitrary units as mean±SEM.

Cell Culture Studies
Aortic VSMCs were isolated from Sprague-Dawley rats as described previously\textsuperscript{19} and passages 3 to 8 were used after phenotyping by staining for muscle-specific α-actin (Dako) and desmin (Boehringer-Mannheim). Cells were treated with Ald (Sigma) and spironolactone (Sigma). All experiments were performed under 24-hour serum-free conditions. Confocal microscopy was performed as described previously.\textsuperscript{19} At least 50 to 80 cells from ≥3 experiments were examined at each treatment point by 2 blinded investigators. Quantification was done with histogram functions using the MRC laser sharp software. The subcellular regions were outlined manually, and mean fluorescence intensities were obtained for the regions delineated with data presented as mean fluorescence intensity in each cell area. Gas 6 antibody (Santa-Cruz) was used in the studies.

Statistics
Data were analyzed by SPSS 13.0 statistical software, and those with a normal distribution (Kolmogorov–Smirnov test) are shown as mean±SEM. Statistical significance was tested by unpaired t test, or, in case of multiple groups, ANOVA (repeated measures as indicated) with posthoc correction according to Bonferroni. A value of \(P<0.05\) was considered statistically significant.

Results

Gas 6 Expression in dTGR
The dTGR have high circulating Ang II and Ald levels.\textsuperscript{20,21} By age 7 weeks they develop renal inflammatory and fibrotic changes that are MR-dependent. Spironolactone treatment results in marked improvement.\textsuperscript{15} We assessed renal protein expression of Gas 6 in dTGR. Gas 6 was upregulated in glomeruli, tubules, and interstitium, as well as in intima and media of hypertrophied vessels, at week 7 compared to age-matched controls, spironolactone protected from increased renal Gas 6 protein expression (dTGR 4.1±0.4, dTGR+Spiro 1.2±0.1, SD 0.9±0.1; dTGR versus dTGR+Spiro with \(P<0.05\), a representative slight is shown in Figure 1).

Ald-Induced VSMC Gas 6 Expression
To determine whether or not Ald can directly induce Gas 6 expression, we stimulated primary VSMCs and assessed protein expression. Compared to unstimulated cells, Ald induced Gas 6 expression in exposed cells as shown by immunohistochemistry and confocal microscopy. At physiological concentration of 1 μmol/L Ald, we observed a time-dependent response with a maximum at 6 hours as follows: Ctr 16±0.8, Ald 2 hours 15±0.7, Ald 6 hours 81±3.4, Ald 24 hours 22±0.7 (all arbitrary units of fluorescence intensity). Using different doses of Ald at 6 hours, we observed Ald-induced Gas 6 upregulation with doses between \(10^{-7}\) and \(10^{-10}\) mol/L. These results were as follows: Ctr 16±0.1, Ald 10^{-7} mol/L 84±3.8, Ald \(10^{-8}\) mol/L 108±4.0, Ald \(10^{-9}\) mol/L 54±1.8, Ald \(10^{-10}\) mol/L 44±1.9, Ald \(10^{-11}\) mol/L 22±1.0, Ald \(10^{-12}\) mol/L 23±1.6 (all arbitrary units of fluorescence intensity). Spironolactone (1 μmol/L, 30 minutes pretreatment) prevented Ald-induced Gas 6 expression (Ald 1 μmol/L) at 6 hours (Figure 2). Similar results were obtained with Western blots (data not shown).

Gas 6 \(-/-\) Mice
To test the direct in vivo relevance of these observations, we next studied Gas 6 \(-/-\) and wild-type mice. DOCA induced hypertension in both Gas 6 \(-/-\) and Gas 6 \(-/-\) mice, and radiotelemetry showed no difference in blood pressure between the groups at any time point (Figure 3). We measured cardiac and body weight in relation to tibia length at the end...
of the study and determined cardiac index. There was no significant difference in cardiac index between untreated Gas 6+/+ or Gas 6−/−. DOCA led to a significant increase of cardiac index in Gas 6+/+ mice, which was not observed Gas 6−/− mice (controls 4.09±0.04, DOCA Gas 6+/+ 4.53±0.03, DOCA Gas 6−/− 4.08±0.03; DOCA Gas 6+/+ versus Gas 6−/− P<0.05). Left ventricular mass (LVM, in mg) was measured with echocardiography after 6 weeks DOCA (Figure 4). Gas 6−/− were protected from DOCA induced increase in LVM (controls 102±5, DOCA Gas 6+/+ 147±7, DOCA Gas 6−/− 112±5; DOCA Gas 6+/+ versus Gas 6−/− P<0.05). Because inflammatory and fibrotic processes are hallmarks of DOCA-induced remodeling, we assessed protein expression of interleukin 6 and fibronectin in the left ventricle (Figure 4). We observed no significant difference between untreated Gas 6+/+ or Gas 6−/−, but an increased expression of both proteins in DOCA treated Gas 6+/+ compared to Gas 6−/− mice (interleukin 6: controls 0.1±0.0, DOCA Gas 6+/+ 10.6±1.7, DOCA Gas 6−/− 4.2±0.2, DOCA Gas 6−/− 2.2±0.3; DOCA Gas 6+/+ versus Gas 6−/− P<0.05; fibronectin: controls 1.2±0.2, DOCA Gas 6+/+ 4.4±0.2, DOCA Gas 6−/− 2.6±0.2, DOCA Gas 6+/+ versus Gas 6−/− P<0.05). Thus, the semiquantitative protein expression data support the in vivo findings, namely that Gas 6 mediated the DOCA-induced inflammation and fibrosis in the heart.

**Figure 2.** Gas 6 protein in vascular smooth muscle cells (VSMCs). Rat aortic VSMCs incubated with 1 nmol/L Ald for 6 hours (n=5; *P<0.05) have an increased Gas 6 protein expression, 1 µmol/L spironolactone prevented Ald-induced Gas 6 upregulation.

**Figure 3.** Blood pressure response to DOCA. Uninephrectomized Gas 6−/− mice and wild-type controls were uninephrectomized. Two weeks later a first 50-mg DOCA pellet (slow release lasting 3 weeks) was implanted, followed by a second pellet after 3 weeks. No difference in blood pressure increase was observed between both strains until reaching blood pressure steady state.

**Figure 4.** Gas 6 knockout protects from DOCA-induced cardiac damage. Left ventricular mass (LVM, in mg±SEM) was measured with echocardiography (n=5 per group) after 6 weeks of DOCA. Gas 6−/− are protected from DOCA-induced increase in LVM (A). Interstitial fibronectin (B) and interleukin 6 (observed mainly in the vasculature and in infiltrating cells; C) expression were increased in Gas 6+/+ mice, and this response was blunted in Gas 6−/− mice.

**Gas 6−/− Mice Are Protected From Renal Injury**

We measured albuminuria in 24-hour urine collections (Figure 5A). Under baseline conditions, there was no significant difference in albumin excretion between the controls, irrespective of the genetic background. DOCA-induced albuminuria (mg/d) was significantly less in Gas 6−/− mice compared to Gas 6+/+ (controls 0.1±0.0, DOCA Gas 6+/+ 10.6±1.7,
DOCA Gas 6/– 5.2±1.5; DOCA Gas 6+/+ versus Gas 6/– P<0.05). Similarly, determining albumin/creatinine ratio in the urine (μg/μmol), we found Gas 6/– to have a reduced quotient (controls 36±7, DOCA Gas 6+/+ 1803±353, DOCA Gas 6/– 717±198, DOCA Gas 6+/+ versus Gas 6/– P<0.05). Renal histopathology demonstrated vascular hypertrophy mainly in small vessels, enhanced glomerular sclerosis, atrophied and hyalinized tubules, as well as increased number of infiltrating cells in DOCA Gas 6+/+ compared to DOCA Gas 6/– and untreated mice (Figure 5; histopathology: controls 0.4±0.2, DOCA Gas 6+/+ 4.4±0.4, DOCA Gas 6/– 2.4±0.2, DOCA Gas 6+/+ versus Gas 6/– P<0.05). To illustrate inflammatory and fibrotic changes in the kidneys we stained the organs for collagen IV and TNF-α. We found no significant difference between the control animals. Under DOCA, Gas 6/– showed less expression of both markers, compared to Gas 6+/+, as observed with immunohistochemistry (Figure 5; collagen IV: controls 1±0.3, DOCA Gas 6+/+ 4.6±0.4, DOCA Gas 6/– 2.8±0.4, DOCA Gas 6+/+ versus Gas 6/– P<0.05; TNF-α: controls 0.6±0.2, DOCA Gas 6+/+ 4.6±0.2, DOCA Gas 6/– 2.2±0.2, DOCA Gas 6+/+ versus Gas 6/– P<0.05). The observed semiquantitative protein expression data obtained from kidneys support our in vivo findings that Gas 6 mediates DOCA-induced inflammation and fibrosis.

**Discussion**

The results of our study demonstrate the pivotal role for Gas 6 in MR-mediated target organ damage. We found that Gas 6 expression was upregulated in a highly activated RAAS rat model. MR blockade reduced this upregulation. We next tested VSMCs in vitro and found that Ald stimulated Gas 6 expression, without the presence of Ang II. We then directed our attention to Gas 6/– mice and observed that the gene-deleted mice were protected from cardiac and renal injury, compared to their wild-type controls. This protection was independent of blood pressure reduction. Our results are consistent with Gas 6 participation in Ald-induced target-organ damage.

Several groups have shown potent protection by blocking Gas 6 action in diverse models of inflammatory diseases. Interestingly, these models also profit from RAAS blockade under clinical conditions. Motoko et al investigated antithrombin nephritis in Gas 6/– and wild-type mice.22 The gene-deleted mice were protected from increased mortality as well as proteinuria and glomerular damage, namely glomerular cell proliferation and sclerosis. Nagai et al induced diabetic nephropathy and found similar protection from streptozotocin-induced diabetic changes in Gas 6/– compared to wild-type mice.23 Gas 6/– mice developed less mesangial hypertrophy, and their renal function was preserved. Lutgens et al intercrossed Gas 6/– with ApoE/– and wild-type mice.24 Gas 6 gene deletion in the crossbred strain resulted in increased plaque stability and reduced plaque inflammation, indicating a role for Gas 6 in the pathology of atherosclerosis and its complications. Tjwa et al compared inflammatory response during a heterotopic mouse model of cardiac transplant injury.8 Gas 6/– mice were
protected from graft loss and did not show signs of myocardial cell death, inflammation, or platelet leukocyte sequestration, compared to wild-type controls. In addition, the group performed crossover transplantation experiments. Gas $6^{-/-}$ hearts were transplanted into wild-type mice and wild-type hearts into Gas $6^{-/-}$ mice. No protection occurred in either experiment, indicating the detrimental effect of Gas 6 in both, as the transplanted heart and the host tissue were both affected. The latter presumably occurred through Gas 6 released from circulating cells or plasma. The observation is interesting in view of the fact that Gas 6 plasma levels were increased in patients with severe septicemia and correlated with disease severity and especially with renal dysfunction. The data suggest that Gas 6 very likely acts as a pathogenic factor in numerous inflammatory disease states. Patients with these diseases might profit from direct and specific pharmacological inhibition of Gas 6.

Inflammation is a key mechanism in the pathophysiology of cardiovascular diseases, and the RAAS plays a pivotal role. Numerous clinical studies support the beneficial effects of RAAS blockade in diverse inflammatory disorders. In the dTGR animal model Gas 6 was overexpressed in tissue with pronounced features of inflammatory remodeling. This state-of-affairs was demonstrated for the kidneys, with similar results in the heart. We characterized these rats earlier and showed a close relationship between inflammatory and immune pathways contributing to the severe target-organ damage. The highly upregulated Gas 6 protein in dTGR, compared to controls, suggested an association between Gas 6 expression, the observed target-organ remodeling, and the RAAS. We next asked the question whether or not our observation was a direct link between RAAS and Gas 6. We observed earlier that Ang II stimulates Gas 6 expression in VSMCs. Cross-talk between Ang II and Ald signaling is well established. Moreover, experimentally and clinically, Ald can act as a major culprit in target-organ damage. We therefore focused on Ald-mediated pathways. We observed a direct stimulating effect on Gas 6 protein expression in VSMCs exposed to Ald. Others have already delineated Gas 6 pathways in vitro. For instance, Gas 6 antiapoptotic effects have been shown in VSMCs, endothelial cells, fibroblasts, and epithelial cells. Furthermore, Gas 6 contributes to inflammatory processes, activates endothelial cells, promotes leukocyte and platelet endothelial sequestration, and participates in vasculitis induction.

To demonstrate a direct causal role of Gas 6 in Ald-induced pathology, we next selected Gas $6^{-/-}$ mice and subjected them to DOCA. DOCA-induced target organ damage is an established and widely used model to investigate mineralocorticoid-induced remodeling. DOCA has mostly been studied in uninephrectomized mice or rats subjected to high salt intake. In this study, we used DOCA for 6 weeks in uninephrectomized mice receiving a normal salt diet. This protocol led to significant blood pressure increases and cardiac and renal injury in wild-type mice. DOCA-treated Gas $6^{-/-}$ mice developed the same degree of hypertension; however, they were substantially protected from target-organ damage.

There is increasing evidence that blood pressure is not solely sufficient to cause cardiac and renal damage. In earlier studies, we described pronounced fibrotic and inflammatory remodeling in dTGR treated with antihypertensive drugs, which did not target the RAAS. Blood pressure is only one contributing risk factor among others in the pathophysiology of target organ damage. Other factors include variants in candidate genes, as was recently shown for the gene encoding nonmuscle myosin heavy chain type II isoform A (MYH9) for nondiabetic end stage renal disease in blacks. Thus, blood pressure requires other synergistically acting cofactors, including elevated blood glucose, high salt intake, latent ischemia associated with vascular remodeling, and other insults that synergistically determine the end result, namely cardiac hypertrophy, renal damage, and vasculopathy.

The complex pathogenic network also influences the development of renal damage and albuminuria. In our experiments, we observed a reduced amount of DOCA-induced albuminuria in Gas $6^{-/-}$ mice, compared to controls. Our results are similar to those reported by Nagai et al. We observed a significantly reduced albuminuria in DOCA-treated Gas $6^{-/-}$ mice, although the reduction was not complete. Nagai et al and our group thus report findings that support a central role for Gas 6 in renal pathophysiology. However, we are aware of interspecies differences and extrapolating these findings to man necessarily requires great caution. A host of other contributing variables, which we can more readily control in animal experiments, influence the human phenotype.

There is increasing evidence that Ang II and Ald act synergistically. Cell culture studies support the fact that Ang II and Ald can potentiate each other in cell proliferation, migration, and altered protein activity. We did not attempt to compare the role of Gas 6 in Ang II–versus Ald-induced pathology. However, from our current knowledge we would speculate that Gas $6^{-/-}$ mice might also be resistant to an Ang II challenge.

Perspectives

Gas 6 expression depends on δ-carboxylation, which is compromised by vitamin K–antagonizing therapy (coumarins). Thus, warfarin or phenprocoumon-treated patient will most likely have reduced Gas 6 action. This state of affairs could be relevant under certain clinical conditions, such as in patients with hyperaldosteronism or septicemia. In addition, other vitamin K–dependent proteins interact with Gas 6 signaling, such as protein S, which could increase the effects of vitamin K antagonists. Further studies will be necessary to elucidate how Gas 6–interfering therapies might best be targeted pharmacologically.

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