Role of Calcitonin Gene-Related Peptide in Hypertension-Induced Renal Damage

Mark C. Bowers, Khurshed A. Katki, Arundhati Rao, Michael Koehler, Parag Patel, Alvin Spiekerman, Donald J. DiPette, Scott C. Supowit

Abstract—Calcitonin gene-related peptide, a potent vasodilator neuropeptide, is localized in perivascular sensory nerves. We have reported that α-calcitonin gene-related peptide knockout mice have elevated baseline blood pressure and enhanced hypertension-induced renal damage compared with wild-type controls. Thus, the aim of this study was to determine the mechanism and functional significance of this increased hypertension-induced renal damage. We previously demonstrated by telemetric recording that the deoxycorticosterone–salt protocol produces a 35% increase in mean arterial pressure in both α-calcitonin gene-related peptide knockout and wild-type mice. Both strains of mice were studied at 0, 14, and 21 days after deoxycorticosterone–salt hypertension. Renal sections from hypertensive wild-type mice showed no pathological changes at any time point studied. However, on days 14 and 21, hypertensive knockout mice displayed progressive increases in glomerular proliferation, crescent formation, and tubular protein casts, as well as the inflammatory markers intercellular adhesion molecule-1, vascular adhesion molecule-1, and monocyte chemoattractant protein-1. There was a significant increase in 24-hour urinary isoprostane, a marker of oxidative stress-induced lipid peroxidation, levels at days 14 and 21 in the hypertensive knockout compared with hypertensive wild-type mice. Urinary microalbumin was significantly higher (2-fold) at day 21 and creatinine clearance was significantly decreased 4-fold in the hypertensive knockout compared with hypertensive wild-type mice. Therefore, in the absence of α-calcitonin gene-related peptide, deoxycorticosterone–salt hypertension induces enhanced oxidative stress, inflammation, and renal histopathologic damage, resulting in reduced renal function. Thus, sensory nerves, via α-calcitonin gene-related peptide, appear to be renoprotective against hypertension-induced damage. 

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Key Words: calcitonin gene-related peptide ■ hypertension ■ kidney

Calcitonin gene-related peptide (CGRP), a 37-amino acid neuropeptide, is derived from the tissue-specific splicing of the primary transcript of the calcitonin (CT)/CGRP gene.1–4 Although CT is produced mainly in the C cells of the thyroid, CGRP synthesis is limited almost exclusively to specific regions of the central and peripheral nervous systems. Immunoreactive CGRP and its receptors are widely distributed in the nervous and cardiovascular systems.1–3 In the peripheral nervous system, prominent sites of CGRP synthesis are the dorsal root ganglia. These structures contain the cell bodies of sensory nerves that terminate peripherally on blood vessels and centrally in laminae I/II of the dorsal horn of the spinal cord.5 A dense perivascular CGRP neural network is seen around the blood vessels in all vascular beds. In these vessels, CGRP-containing nerves are found at the junction of the adventitia and the media passing into the muscle layer.1 Receptors for CGRP have been identified in the media, intima, and endothelial layer of resistance vessels.1–4 CGRP is the most potent vasodilator discovered to date and it has positive chronotropic and inotropic effects.5,6 CGRP has been shown to selectively dilate multiple vascular beds, with the coronary vasculature being a particularly sensitive target.6,7 In the kidney, α-CGRP has been shown to increase renal blood flow and the glomerular filtration rate, thus enhancing natriuresis and diuresis.7–10 Stimulation of CGRP receptors in the kidney reduces renovascular resistance and relaxes the afferent arterioles in the glomeruli.9,10 Systemic administration of CGRP decreases blood pressure (BP) in a dose-dependent manner in normotensive and hypertensive animals and humans.1,5,6,11 The primary mechanism responsible for this reduction in BP is peripheral arterial dilation.

A direct role for CGRP in experimental hypertension has been established. CGRP can significantly attenuate the pathological effects of chronic hypoxic pulmonary hypertension.12 Furthermore, we have reported that CGRP plays a compensatory vasodilator role to attenuate the BP increase in 3 models of experimental hypertension: deoxycorticosterone
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P expression, and BP phenotype have been described previously.21,22 After a 2-week recovery period, the DOC-salt protocol was initiated. For this procedure, the mice were anesthetized with ketamine:xylazine (80 mg/kg:4 mg/kg). One kidney was removed and a 50-mg DOC pellet (21-day release; Innovative Research of America) was implanted subcutaneously in the left side of the abdomen. The DOC-salt mice were placed on 0.9% saline drinking water. Two control groups, consisting of α-CGRP KO and WT mice (n=6 each), were sham-operated, placebo pellet-implanted, and given tap water to drink. After surgery, radiotelemetric data were recorded for an additional 3 weeks.21,22

Histopathology and Immunohistochemistry
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Urinary Lipid Peroxidation Assays
An enzyme immunoassay for urinary isoprostane (Oxford Biomedical, Oxford, Mich) was used to analyze free radical-mediated peroxidation of lipoproteins. Samples were collected in metabolic cages for 24-hour intervals at days 0, 14, 21. The mice were placed in the metabolic cages for 2 days to adapt to the metabolic cages. This assay was performed according to the manufactures instructions and completed in triplicate. This assay was validated by performing a modified urinary MDA [malonaldehyde bis-(dimethyl acetal)] lipid peroxidation assay.23

Urinary and Plasma Analyses
The 24-hour urine samples were collected from the mice in metabolic cages 21 days after initiation of the DOC-salt protocol. Blood was drawn before euthanization, samples were centrifuged at 4000g for 10 minutes at 4°C, and the plasma was collected. Urine creatinine, plasma creatinine, and total urinary protein concentrations were analyzed by the Clinical laboratory at the Scott & White Health System using a Dade Behring RxL Dimension Analyzer and Reagents. Creatinine clearance, in microliters of plasma and urine per minute was calculated by creatinine clearance \( C_{cr} = (C_u/C_p) \times V \), where \( C_u \) is the concentration of creatinine in urine, \( C_p \) is the concentration of creatinine in plasma at the time of a 24-hour urine collection, and \( V \) is the urine flow rate in microliters per minute. Microalbumin was quantified using a marine microalbuminuria enzyme-linked immunosorbent assay (Exocell Inc, Philadelphia, Pa). The assays were performed as recommended by the supplier.

Statistical Analysis
Statistical significance was determined by the Student \( t \) test or when appropriate by ANOVA followed by the Tukey–Kramer multiple comparisons test. The acceptable level of significance was \( P<0.01 \).

Results

Gross Postmortem and Histopathologic Examination
Although the α-CGRP KO mice appear to have a normal phenotype,17 with the exception of an elevated basal MAP,18,21 before using these animals for histopathologic studies after induction of hypertension with the DOC-salt protocol, a comprehensive pathological evaluation was performed to determine if there were any significant developmental or pathological changes in the absence of treatment. No significant gross postmortem or histopathologic alterations were detected in the body cavities, or integumentary, alimentary, respiratory, circulatory, urogenital, endocrine, hematopoietic, musculoskeletal, and nervous systems of the α-CGRP KO mice compared with their WT counterparts. The only exception was a modest, but significant, increase in the heart-to-body weight ratio in the α-CGRP KO mice, which is likely the result of the elevated BP. In addition, there was no microscopic evidence of vascular alterations or vascular variations among the mice examined.

Mean BP Measurements
As previously reported, basal average 24-hour MAP, as determined by telemetric recording, was significantly higher in the α-CGRP KO (120±3 mm Hg) compared with WT (107±3 mm Hg) controls.21 After initiation of the DOC-salt protocol, the BP increased over 4 days in both groups to final values of 166±5 mm Hg for the α-CGRP KO and 147±4 mm Hg for the WT mice. There was no significant difference in body weights in any of the groups studied either at the beginning or end of the protocol. The rate of BP increase was not appreciably different between the 2 DOC-salt groups and the ∼20 mm Hg BP differential was observed during the day and night.21 When normalized to baseline BP,
this represents an ≈35% increase in MAP for the 2 groups. The MAP was unchanged in the 2 control groups. Both the α-CGRP KO and WT mice displayed a normal 24-hour circadian rhythm, both before and after DOC-salt treatment. The average heart rate tended to be higher in the α-CGRP KO mice but was not statistically significant.21

**Histopathologic Study of the Hypertensive and Control α-CGRP KO and WT Mice**

After the BP measurement studies, the mice were euthanized and the kidneys were removed for histopathologic examination. As before, no changes were seen in the kidneys between the control α-CGRP KO and WT mice. Furthermore, no significant pathological changes were seen in the kidneys from the DOC-salt hypertensive WT mice (Figure 1) compared with their normotensive controls. In contrast, marked damage was evident in the kidney sections from the DOC-salt hypertensive α-CGRP KO mice. The kidneys of these mice showed moderate 2+ glomerular changes, including congestion of the capillary loops, focal mesangial proliferation, crescentic proliferation, and focal histiocytic infiltration (Figure 1). Proteinaceous casts were also noted in a number of tubules.

**Immunohistochemical Markers of Renal Inflammation**

On a scale of 0 to +4, there was a significant and time-dependent progressive increase in the expression of the inflammatory markers, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule (VCAM)-1, and monocyte chemoattractant protein-1 (MCP-1) in the DOC-salt α-CGRP KO mice, whereas only minimal alterations were observed in the DOC-salt WT mice (Table 1). The hypertensive α-CGRP KO mice showed an increase in ICAM-1 expression from 0 to +3, VCAM-1 (0 to +2), and MCP-1 (0 to +3) over the indicated time points. Hypertensive WT mice showed an increase in ICAM-1 expression from 0 to +2, VCAM-1 (0 to +1), and MCP-1 (0 to +1). Representative sections of this analysis are shown in Figure 2, which demonstrates positive immunohistochemical staining of kidney sections taken from day 21 α-CGRP and WT DOC-salt mice. There was some, but minimal, expression of ICAM-1 (+1) in the α-CGRP KO control mice at day 21.

**Urinary Lipid Peroxidation in the DOC-Salt Hypertensive α-CGRP KO and WT Mice**

There were significant increases in urinary biomarkers of lipid peroxidation between control and DOC-salt–treated mice, as well as between α-CGRP KO DOC-salt and WT DOC-salt mice, as shown in Figure 3. Using a urinary isoprostane assay, it was shown at day 21 that there was a 4-fold and 6-fold increase in urinary isoprostane levels in the DOC-salt–treated WT (day 0, 122±1 pg/mL per 24 hours; day 21, 540±18 pg/mL per 24 hours) and α-CGRP KO (day 0, 125±2 pg/mL per 24 hours; day 21, 725±23 pg/mL per 24 hours) mice, respectively (Figure 3A). Likewise at day 21, there was a 3-fold and 5-fold increase in urinary MDA levels in the DOC-salt–treated WT and α-CGRP KO mice, respectively, with a significant difference between the α-CGRP KO and WT mice (68±3 nmol MDA equivalents/mL versus 50.7±1.9 nmol MDA equivalents/mL; P<0.01; Figure 3B).

**Microalbumin and Total Protein in Hypertensive α-CGRP KO and WT Mice**

As expected, the DOC-salt protocol produced ≈10-fold increase in urinary output in both the α-CGRP KO and WT mice (Table 2). DOC-salt treatment also produced a 2.5-fold and 7.0-fold increase in microalbumin excretion in the DOC-salt–treated WT and α-CGRP KO mice, respectively, with the excretion in the α-CGRP KO mice being significantly greater than the WT mice. Table 2 also shows a 1.3- and 2.7-fold increase in total urinary protein concentrations with the DOC-salt treatment in both the α-CGRP KO mice and WT mice, respectively, with the protein excretion in the hypertensive α-CGRP KO mice being significantly greater than the WT mice.

**TABLE 1. Analysis of Immunohistochemical Markers of Renal Inflammation**

<table>
<thead>
<tr>
<th>Inflammatory Markers</th>
<th>ICAM-1</th>
<th>VCAM-1</th>
<th>MCP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>0</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>+1</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CGRP KO control n=5</td>
<td>0</td>
<td>0</td>
<td>+1</td>
</tr>
<tr>
<td>CGRP KO DOC-salt n=5</td>
<td>0</td>
<td>+2</td>
<td>+3</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>+1</td>
<td>+2</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>+1</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>+1</td>
</tr>
<tr>
<td>WT control n=4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>WT DOC-salt n=4</td>
<td>0</td>
<td>+1</td>
<td>+2</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>+1</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>+1</td>
</tr>
</tbody>
</table>

Scoring system was based on subjective analysis of immunohistochemical staining. Staining was ranked from 0 to +4, with 0 representing no staining and +4 representing maximum and diffuse staining.
Creatinine Clearance in the DOC-Salt Hypertensive α-CGRP KO and WT Mice

DOC-salt treatment produced a 4.5-fold and 6.4-fold decrease in creatinine clearance in the DOC-salt–treated WT and α-CGRP KO mice, respectively. The creatinine clearance was significantly lower in the DOC-salt–treated α-CGRP KO mice compared with the DOC-salt–treated WT mice. Creatinine clearance was also significantly lower between control α-CGRP KO mice compared with control WT mice (Table 2).

Discussion

The significant findings of this study are: (1) DOC-salt hypertensive α-CGRP KO mice displayed markedly enhanced and progressive renal damage and expression of the inflammatory markers ICAM-1, VCAM-1, and MCP-1 compared with the DOC-salt–treated WT mice; and (2) consistent with the histopathologic changes, the DOC-salt α-CGRP KO mice exhibited significantly elevated urinary biomarkers of oxidative stress and a marked reduction in kidney function as evidenced by increased total protein and microalbumin levels in the urine and decreased creatinine clearance compared with the DOC-salt WT mice. The marked difference in the degree of renal damage, between the α-CGRP KO DOC-salt mice and the WT DOC-salt mice, suggests that the absence of α-CGRP in perivascular sensory nerves contributes to the pathological changes observed. There are 3 possible mechanisms that underlie these observations. The first is the higher absolute MAPs in the DOC-salt α-CGRP KO mice compared with their WT counterparts because of the loss of a potent vasodilator system. The second is caused by a direct effect of the lack of α-CGRP. The third is that permanent deletion of the α-CGRP gene may lead to alterations in other neuro-humoral systems that may influence BP and hypertension-induced end-organ damage.

Evidence for a primary role for the absolute higher MAP in the DOC-salt α-CGRP mice compared with the DOC-salt WT animals is provided in a recent study that was designed to quantify the relative contribution of BP versus angiotensin II on kidney damage in a rat model of angiotensin II–induced hypertension. This report demonstrated that the preferential juxtedudillary damage in this setting is largely induced by BP and is probably mediated through the transforming growth factor-β and NF-κB pathway. It should also be noted that although the DOC-salt–treated α-CGRP KO mice are clearly in the hypertensive range, their WT counterparts are in the borderline range. By analogy with humans, little vascular damage might be expected with borderline hypertension, whereas considerably greater damage might be expected with frank hypertension.

Alternatively, the arguments supporting a direct effect of the absence of α-CGRP are: (1) basal BP is 10 to 15 mm Hg higher in the α-CGRP KO compared with WT controls in the absence of any pathological changes in the kidneys between the 2 groups; (2) the DOC-salt protocol produces ≈40 mm Hg increase in the MAP of the WT mice, again without any significant histopathologic damage to renal tissues, although kidney dysfunction is present as shown by an increase in urinary total protein and microalbumin concentrations; and (3) the dramatic differences in the degree of kidney damage and the significant increase in markers of oxidative stress and inflammation between the DOC-salt treated α-CGRP KO and WT mice. However, we do not know whether the correlation between MAP and pathological changes is linear. In addition, the BP difference between 120 and 107 mm Hg (basal MAPs of α-CGRP KO and WT mice) is not likely the same as that between 166 and 147 mm Hg (final MAPs of the hypertensive α-CGRP KO and WT mice).

Finally, deletion of the α-CGRP gene may also indirectly augment hypertension-induced end-organ damage. It is known that that α-CGRP KO mice display significantly enhanced activity of the sympathetic nervous system and...
Although at this point we cannot differentiate between the 3 potential mechanisms discussed, there is considerable evidence that increased reactive oxygen species (ROS) initiates the inflammatory responses observed in a number of cardiovascular disease states including hypertension-induced end-organ damage. Oxidative stress and ROS have been demonstrated in several animal models of hypertension including spontaneous hypertension,\(^2\) renovascular hypertension,\(^28\) DOC-salt–induced hypertension,\(^29\) and obesity-related hypertension.\(^30\) DOC-salt hypertension produced a significant increase in urinary markers of lipid peroxidation in both the \(\alpha\)-CGRP KO and WT mice; however, the increase was much more pronounced in the \(\alpha\)-CGRP KO mice. Oxidative stress leads to activation of transcription factors such as NF-\(\kappa\)B, which in turn activate genes that trigger inflammation, including ICAM-1, VCAM-1, and MCP-1. The presence of all 3 markers was detectable at day 14 in the DOC-salt \(\alpha\)-CGRP KO mice. This inflammatory response was progressive and by day 21 there was marked inflammation as seen by the localization and staining intensity in comparison to the absence or minimal expression of these markers in DOC-salt WT mice. However, we do not know at this point whether increase ROS initiated the damage or was simply a response to the injury.

These results described correlated well with the marked increase in urinary total protein and microalbumin, as well as the decrease in the creatinine clearance in the hypertensive \(\alpha\)-CGRP KO mice. There was, however, at day 21, a significant increase in total protein and urinary microalbumin excretion in the DOC-salt–treated WT mice compared with normotensive WT controls that is indicative of early renal damage. At day 21, there was also a significant decrease in creatinine clearance in the DOC-salt–treated WT mice that is again a clear sign of renal dysfunction. Interestingly, the \(\alpha\)-CGRP KO control mice had a significant reduction in creatinine clearance compared with the \(\alpha\)-CGRP KO groups and WT groups.

### Table 2. Urinary Markers of Renal Function in \(\alpha\)-CGRP KO and WT Mice

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Urinary Output, mL/24 h</th>
<th>Urinary Total Protein, mg/mL</th>
<th>Creatinine Clearance, (\mu)L/ml</th>
<th>Urinary Microalbumin, (\mu)g/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (\alpha)-CGRP KO n=12</td>
<td>1.3±0.2</td>
<td>559±94</td>
<td>110±12.7†</td>
<td>4.9±1.5</td>
</tr>
<tr>
<td>DOC-salt (\alpha)-CGRP KO n=10</td>
<td>14.8±2.1†</td>
<td>1524±306†</td>
<td>17.2±2.0†</td>
<td>34.2±3.3†</td>
</tr>
<tr>
<td>Control WT n=5</td>
<td>2.1±0.6</td>
<td>633±88</td>
<td>331.1±20.0</td>
<td>6.6±1.0</td>
</tr>
<tr>
<td>DOC-salt WT n=7</td>
<td>20.2±2.0†</td>
<td>850±117†</td>
<td>73.4±3.5†</td>
<td>15.9±0.5†</td>
</tr>
</tbody>
</table>

Values were collected in metabolic cages during a 24-hour time interval at day 21. Values are reported as the mean±SEM.

\(\ddagger\) \(P<0.01\) between control and experimental DOC-salt groups.

\(\ddagger\) \(P<0.01\) between DOC-salt, \(\alpha\)-CGRP KO, and WT groups.

\(\ddagger\) \(P<0.01\) between \(\alpha\)-CGRP KO and WT controls.
uria. The most likely reason that we did not observe any changes using light microscopy is that the early pathological changes require more sensitive methods of detection such as electron microscopy and acute biochemical markers of cellular change/remodeling since one would not expect rapid and extensive damage to occur in a 3-week time period.

To our knowledge, this is the first report that demonstrates, in a mouse model of hypertension, that the absence of α-CGRP significantly exacerbates renal oxidative stress and inflammation, in conjunction with renal end-organ damage and marked dysfunction. In a model of vasoconstrictive ischemia and reperfusion, evidence indicates that isoprostane, at levels similar to those we observed, can induce the rapid adhesion of polymorphonuclear neutrophils, a condition leading to inflammation and oxidative stress-induced tissue damage. Reports suggest that CGRP can inhibit both neutrophil adhesion and platelet aggregation to endothelial cells, thereby ameliorating endothelial dysfunction and renal injury.1,2

Indirect evidence of a role for α-CGRP in end-organ protection, through the direct attenuation of ROS, is provided by studies of the peptide, adenomedullin. Adrenomedullin is a member of the calcitonin/CGRP gene family, binds to the CGRP receptor, and has been shown to attenuate hypertension-induced oxidative end-organ damage. It has also been suggested that the protective effects of adrenomedullin is mediated through the inhibition of NADPH oxidase activity. Therefore, the deletion of α-CGRP may significantly enhance renal vasoconstriction and reduce renal perfusion, thereby exacerbating the damage subsequent to increased generation of inflammatory mediators and ROS.

Perspectives
These data demonstrate that the deletion of the α-CGRP gene enhances hypertension-induced end-organ renal damage. The mechanism of this increased tissue damage may be through the loss of α-CGRP–mediated vasodilator activity, resulting in higher BP and reduced renal blood flow, leading to an increase in local tissue production of oxidative and inflammatory mediators or the loss of a direct protective effect of α-CGRP, among others. This is the first report to our knowledge of a sensory nerve-mediated renal protective effect against hypertension-induced end-organ damage. Traditionally, sensory nerves were defined as purely afferent neurons that monitor changes in their chemical and physical environment and convey this information to the central nervous system. The capacity to act in an efferent manner is mediated by the release of neuropeptides, including α-CGRP, from their peripheral terminals that regulate vasodilation and other activities independently of sensation. Thus, this organ-protective activity of α-CGRP may reflect another significant function of the efferent arm of the sensory nervous system.

Acknowledgments
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References


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