Renal protective effects of α-calcitonin gene-related peptide in deoxycorticosterone-salt hypertension

Jianping Li, Kevin A. Carnevale, Donald J. DiPette, and Scott C. Supowit

1Department of Cell Biology and Anatomy, University of South Carolina School of Medicine, Columbia, South Carolina; 2Department of Pathology, Microbiology, and Immunology, University of South Carolina School of Medicine, Columbia, South Carolina; and 3Department of Medicine, University of South Carolina School of Medicine, Columbia, South Carolina

Submitted 1 August 2012; accepted in final form 1 February 2013

Li J, Carnevale KA, DiPette DJ, Supowit SC. Renal protective effects of α-calcitonin gene-related peptide in deoxycorticosterone-salt hypertension. Am J Physiol Renal Physiol 304: F1000–F1008, 2013; doi:10.1152/ajprenal.00434.2012.—Deoxycorticosterone salt (DOC-salt) hypertension-induced renal damage is enhanced in α-calcitonin gene-related peptide (α-CGRP) knockout (KO) compared with wild-type (WT) mice. However, since the α-CGRP KO mice have a 15–20 mmHg higher baseline mean arterial pressure (MAP) than WT mice, they also have a higher MAP than WT mice throughout the course of the DOC-salt hypertension. To determine the mechanism by which the absence of α-CGRP enhances hypertension-induced renal damage, DOC-salt hypertension was induced in telemetry probe implanted α-CGRP KO and WT mice. To equalize the blood pressure (BP) to that of DOC-salt WT mice, an additional group of DOC-salt α-CGRP KO mice was given 0.025% hydralazine to drink. The DOC-salt protocol increased the final MAP in α-CGRP KO mice to 155 ± 6 mmHg and in WT mice to 140 ± 5 mmHg. The MAP of the hydralazine-treated DOC-salt α-CGRP KO mice was 139 ± 6 mmHg. Urinary excretion of microalbumin and isoprostane, a marker for oxidative stress, was increased, and creatinine clearance was decreased in DOC-salt α-CGRP KO compared with DOC-salt WT mice. Equalization of the MAP in DOC-salt α-CGRP KO to that of DOC-salt WT mice did not significantly improve these parameters. Renal macrophage infiltration; desmin, a marker of podocyte damage; and the inflammatory cytokines TNF-α and IFN-γ and the chemokines monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) were increased in DOC-salt α-CGRP KO mice and were not reduced by hydralazine treatment. However, BP equalization did improve the renal histopathological damage, as determined by light microscopy. Therefore, in DOC-salt hypertension in mice, the mechanism(s) of the renal protective effects of α-CGRP are both BP independent and BP dependent.

sensory nervous system; neuropeptides; knockout mice; inflammation

THE SENSORY NEUROPEPTIDE α-calcitonin gene-related peptide (α-CGRP) has been shown to play a significant role in the regulation of cardiovascular function (3, 4, 22, 24, 33). Administration of CGRP decreases blood pressure (BP) in a dose-dependent manner in normotensive and hypertensive animals and humans primarily through peripheral arterial dilation (3, 4, 22, 24, 33). Stimulation of CGRP receptors in the kidney relaxes the afferent arterioles and increases renal blood flow and the glomerular filtration rate (3, 30, 31).

CGRP is known to play a counterregulatory role in several models of experimental hypertension. We have demonstrated that CGRP attenuates the increased BP in deoxycorticosterone (DOC-salt; Ref. 27), subtotal nephrectomy (SN-salt; Refs. 25, 28), and l-NAME-induced hypertension during pregnancy (7). This antihypertensive activity of CGRP is mediated by an upregulation of neuronal CGRP synthesis and release and/or enhanced sensitivity of the peripheral vasculature to the vaso-dilator effects of this neuropeptide (7, 25, 27, 28).

Using the α-CGRP knockout (KO) mouse, our laboratory has demonstrated by long-term telemetric monitoring that the deletion of α-CGRP results in a significant elevation in the basal BP (8). We have also demonstrated that deletion of the α-CGRP gene enhances DOC-salt hypertension-induced end organ damage in the heart and kidney and that this protective effect is mediated, in part, through the reduction of oxidative stress and inflammation (2, 26). In these previous studies, even though the DOC-salt protocol produced an equal increase in BP (calculated as a percent increase from baseline) in the α-CGRP KO and WT mice, the absolute BP was higher in the α-CGRP KO animals because of the increased basal BP. Therefore, the purpose of the present study was to determine whether the mechanism by which the absence of α-CGRP results in enhanced renal damage in DOC-salt hypertension is by BP-dependent and/or BP-independent mechanisms. To accomplish this objective, three groups of telemetry implanted mice were studied: DOC-salt WT, DOC-salt α-CGRP KO, and DOC-salt α-CGRP KO mice treated with hydralazine. Kidneys, blood, and urine from the experimental and control groups were then analyzed.

METHODS

Induction of DOC-salt hypertension in α-CGRP KO and WT mice. All experiments were approved by the University Animal Care and Use Committee and were consistent with the ethical guidelines of the National Institutes of Health. Ten-week-old male α-CGRP KO mice on a C57/BL6 genetic background and C57/BL6 WT mice (Charles River) were used for this study (n = 6–9/group). The mice were anesthetized with isoflurane, and the radiotelemetric catheters were surgically implanted. After a 1-wk recovery period, the left kidneys of the mice were removed and a 50-mg DOCA pellet (21-day release; Innovative Research of America) was implanted. On day 22, after implantation of the first pellet, another 50-mg DOCA pellet was implanted. The mice were placed on 0.9% NaCl drinking water following DOCA pellet implantation. One group of DOC-salt α-CGRP KO mice was given hydralazine (0.025%) in 0.9% NaCl drinking water to equalize the MAP to that of the DOC-salt WT mice. The dose of hydralazine was adjusted daily based on the 24-h MAPs (moving averages). This treatment regimen was continued for 28 days. BP was recorded after implantation of radiotelemetry (10 s/10 min). Two control groups, consisting of α-CGRP KO and WT mice (n = 6/group), were sham operated, placebo pellet implanted, and given tap water to drink. The BP in the two control groups was
monitored by a tail-cuff blood pressure analysis system (MC4000; Hatteras Instruments, Cary, NC).

Urine and plasma analyses. Twenty-four-hour urine samples were collected from the mice in metabolic cages, and blood was collected at the end of the protocol. Urine and plasma creatinine levels were measured using a creatinine assay kit (Biovision, Mountain View, CA). Creatinine clearance was calculated as described elsewhere (2). Microalbumin was quantified using a murine microalbuminuria ELISA kit (Exocell, Philadelphia, PA). An enzyme immunoassay for urinary isoprostane (Oxford Biomedical, Oxford, MI) was used to analyze free radical mediated peroxidation of lipoproteins.

Histopathology. At the end of the DOC-salt protocol, the animals were anesthetized with ketamine/xylazine and then killed. Kidneys were removed and fixed in PBS containing 4% paraformaldehyde, dehydrated, embedded in paraffin, and cut into 5-μm thick sections. These were stained with Masson’s trichrome. Semiquantitative methods were used to determine the glomerulosclerosis index (GSI), examining 50 glomeruli/kidney, and cortical tubulointerstitial injury scores as described elsewhere (30).

Renal macrophage infiltration and desmin staining. Infiltration of monocytes/macrophages was examined by immunohistochemistry. A monoclonal antibody (rat anti-mouse, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) against the F4/80 that is solely expressed on the surface of macrophages was used. Positive stains were quantified using image analysis software (Image Pro-Plus; Media Cybernetics, Silver Spring, MD) by scanning six nonoverlapping fields of cortex and medulla, respectively, at ×200 magnification for each kidney section and positive areas are expressed as a percentage of total area. Desmin staining was performed as described elsewhere (12). Fifty glomeruli per kidney were analyzed at a magnification of ×400 using a monoclonal antibody against desmin (D33, 1:100; Santa Cruz Biotechnology) A podocyte injury score was given to each glomerulus, which reflected the extent of desmin staining and the percentage of the positive staining area in the glomerular tuft: 0, 0–5% of the area stained; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, >75%. The podocyte injury score for each kidney was defined as the mean desmin score of 50 glomeruli.

Cytokines/chemokines protein assay. To examine inflammatory markers in the kidneys of the mice, protein levels of IL-1β, IL-6, IL-10, IFN-γ, TNF-α, macrophage inflammatory protein-1 (MIP-1α), and monocyte chemotactic protein-1 (MCP-1) were measured by Bio-Plex Pro Assays (Bio-Rad Laboratories, Hercules, CA). Kidney samples were prepared using a Bio-Plex Cell Lysis Kit as recommended by the supplier. To quantify the results, samples were analyzed by a Bio-Rad Bio-Plex System using Bio-Plex Manager 4.0 software.

6-Keto-prostaglandin F1α assay. 6-Keto-prostaglandin F1α (6-keto-PGF1α) is a stable hydrolyzed product of prostacyclin (PGI2), which is rapidly degraded. To determine the concentration of 6-keto-PGF1α in the kidney, an ELISA kit (Neogen, Lexington, KY) was employed. The concentration of 6-keto-PGF1α was adjusted to total protein.

Statistical analysis. Data are presented as means ± SE. Statistical comparison between groups was performed by one-way ANOVA, followed by Tukey’s multiple comparison test analysis. Differences were considered statistically significant at probability values <0.05.

RESULTS

Equalization of blood pressure in the DOC-salt α-CGRP KO mice. As shown in Fig. 1A, the basal average 24-h MAP was significantly higher in the α-CGRP KO compared with the WT controls as we have reported previously (2, 26). After the initiation of the DOC-salt protocol, the overall (28 day) MAP values were 153 ± 6 mmHg for the α-CGRP KO and 138 ± 5 mmHg for the WT mice (Fig. 1A). Hydralazine treatment equalized MAP in the DOC-salt α-CGRP KO mice to that of the DOC-salt WT mice for a value of 133 ± 7 mmHg. When the 28-day average systolic BPs were assessed (Fig. 1B), the values were 182 ± 4, 167 ± 4, and 161 ± 5 mmHg for the three groups described above. The 28-day average MAPs were also calculated for both the nocturnal and diurnal periods. During their nighttime active state, the MAPs for the DOC-salt
control sham-treated both before and after DOC-salt treatment. The BPs of the lower than that of the DOC-salt WT mice. Both the MAP of the hydralazine-treated mice was significantly 
col. The basal BP in the measured every third day before and during the 28-day proto-
duced a significant decrease in creatinine clearance (WT, 70 ± 29, 14.1 ± 2.3 μg·ml⁻¹·24 h⁻¹) or α-CGRP KO (day 0, 6.9 ± 1.5 vs. day 28, 9.4 ± 1.8 μg·ml⁻¹·24 h⁻¹) mice. Urinary levels of isoprostane, a prostaglandin-like compound that is produced by free radical mediated peroxidation of lipoproteins, was used as a measurement of oxidative stress. DOC-salt treatment caused a significant increase in urine isoprostane levels in DOC-salt α-CGRP KO and DOC-salt WT (0.75 ± 0.07 and 0.46 ± 0.11 ng·24 h⁻¹·g body wt⁻¹, respectively) mice compared with controls (α-CGRP KO, 0.21 ± 0.02 and WT, 0.23 ± 0.03 ng·24 h⁻¹·g body wt⁻¹) with the urinary isoprostane level being significantly greater in DOC-salt α-CGRP KO than in DOC-salt WT mice (Fig. 3). Equalization of BP in DOC-salt α-CGRP KO mice by hydralazine did not decrease the urine isoprostane levels (0.72 ± 0.04 ng·24 h⁻¹·g body wt⁻¹).

Renal macrophage infiltration and desmin expression. DOC-salt hypertension significantly increased macrophage infiltration in the kidneys of the α-CGRP KO (4.6 ± 0.4%) and WT (2.1 ± 0.2%) mice compared with the control sham-operated groups (α-CGRP KO, 0.5 ± 0.1% and WT, 0.4 ± 0.1%; Fig. 4). Equalization of the BP in the DOC-salt α-CGRP KO by hydralazine to that of the DOC-salt WT mice did not significantly reduce macrophage infiltration (4.0 ± 0.2%). Podocyte damage was analyzed by desmin staining. DOC-salt hypertension significantly increased desmin expression in glomerular podocytes in the kidneys of the DOC-salt WT (0.32 ± 0.03) mice compared with both control groups of WT (0.04 ± 0.02) and α-CGRP KO (0.05 ± 0.02) mice. Importantly, desmin expression was significantly increased in the DOC-salt α-CGRP KO (0.52 ± 0.04) compared with the DOC-salt WT (0.32 ± 0.03) mice. Equalization of BP in the DOC-salt α-CGRP KO by hydralazine to that of the DOC-salt WT mice

α-CGRP KO, DOC-salt WT, and hydralazine-treated DOC-salt α-CGRP KO mice were 165 ± 6, 151 ± 7, and 144 ± 6 mmHg, respectively. During the daytime inactive state, the overall MAPs were 141 ± 5, 125 ± 6, and 120 ± 6 for the three groups of mice. In addition, since an accurate and thorough assessment of the BP profiles for each of the three groups is critical for this study, statistical analysis was performed for each individual time point (each 24-h daily average) for the groups shown in Fig. 1. The daily MAP values for the DOC-salt α-CGRP mice were significantly higher than those for the DOC-salt WT mice. The daily MAP values for the DOC-salt WT and the hydralazine-treated DOC-salt α-CGRP KO mice were similar except for days 9, 15, 20, and 29 where the MAP of the hydralazine-treated mice was significantly lower than that of the DOC-salt WT mice. Both the α-CGRP KO and WT mice displayed a normal 24-h circadian rhythm, both before and after DOC-salt treatment. The BPs of the control sham-treated α-CGRP KO and WT mice were not telemetrically recorded; however, tail-cuff sphygmomanometric BPs were measured every third day before and during the 28-day protocol. The basal BP in the α-CGRP KO mice was ~15 mmHg higher than in the WT over the 4-wk period. At the end of the protocol, the systolic BPs were 131 ± 5 and 115 ± 4 mmHg for the α-CGRP KO and WT mice, respectively.

Creatinine clearance, urinary microalbumin, and isoprostane. At the end of the 28-day protocol, DOC-salt hypertension produced a significant decrease in creatinine clearance (WT, 70 ± 17 and α-CGRP KO, 18 ± 14 μl/min) and an increase in urinary microalbumin excretion in the DOC-salt-treated α-CGRP KO (657 ± 46 μg/24 h) and WT (326 ± 61 μg/24 h) mice, respectively, with the creatinine clearance being signifi-

Fig. 2. Hydralazine treatment does not improve kidney function in the DOC-salt α-CGRP KO mice. Urine samples (24 h) were collected in metabolic cages at the end of the DOC-salt protocol and plasma samples were taken when the mice were euthanized. Bar graphs show the urinary microalbumin (A) and creatinine clearance (B) from the WT sham (WT), α-CGRP KO sham (KO), DOC-salt WT (DOC WT), DOC-salt α-CGRP KO (DOC KO), and hydralazine-treated DOC-salt α-CGRP KO (DOC KO HYD) groups (n = 6/group) of mice. Values are given as the means ± SE. *P < 0.001 vs. WT and KO; +P < 0.01 vs. DOC WT.

Fig. 3. Hydralazine does not significantly improve renal oxidative stress in the DOC-salt α-CGRP KO mice. Bar graphs show the levels of urinary isoprostane in the WT sham (WT), α-CGRP KO sham (KO), DOC-salt WT, (DOC WT), DOC-salt α-CGRP KO (DOC KO), and hydralazine-treated DOC-salt α-CGRP KO (DOC KO HYD) groups (n = 6/group) of mice. Values are given as the means ± SE. *P < 0.05 vs. WT and KO; +P < 0.05 vs. DOC WT.
did not reduce desmin expression in glomerular podocytes (0.48 ± 0.04; Fig. 5).

Renal cytokines and chemokines. DOC-salt hypertension significantly increased the levels of the proinflammatory cytokines IL-1β, IL-6, TNF-α, and IFN-γ (Fig. 6A) and the chemokines MCP-1 and MIP-1α (Fig. 6B) in the kidneys of both the DOC-salt α-CGRP KO and DOC-salt WT mice, with the increases in the levels of TNF-α, IFN-γ, MCP-1 and MIP-1α being greater in the DOC-salt α-CGRP KO than in the DOC-salt WT mice. The anti-inflammatory cytokine IL-10 was significantly increased in both the DOC-salt α-CGRP KO and DOC-salt WT mice, with the increase being significantly less in the DOC-salt α-CGRP KO than in the DOC-salt WT mice. Equilization of the BP by hydralazine in the DOC-salt α-CGRP KO to that of the DOC-salt WT mice did not significantly attenuate the increases in TNF-α, IFN-β, MCP-1, and MIP-1α nor did it increase the level of IL-10. Hydralazine treatment did, however, lower the levels of IL-6.

Kidney 6-keto-PGF₁α content. At the end of the DOC-salt protocol, 6-keto-PGF₁α levels in the renal cortex were significantly increased in the DOC-salt WT mice (0.38 ± 0.02 ng/mg total protein) but not in the DOC-salt α-CGRP KO mice (0.23 ± 0.03 ng/mg total protein), which was similar to levels seen in the WT and α-CGRP KO controls (0.17 ± 0.03 and 0.18 ± 0.02 ng/mg total protein, respectively). Equalization of the BP by hydralazine in DOC-salt α-CGRP KO to that of the DOC-salt WT mice did not increase the levels of the 6-keto-PGF₁α (0.26 ± 0.03 ng/mg total protein; Fig. 7).

Histopathology. The kidneys in both WT and α-CGRP KO sham groups were histologically unremarkable. (Fig. 8, A and B). The renal histopathological changes in the DOC-salt α-CGRP KO mice were more severe than that seen in the DOC-salt WT mice with severe pathological changes present in 50% of the kidneys in the DOC-salt α-CGRP KO animals (4 out of 8 animals) compared with 22% of the kidneys from the animals in the DOC-salt WT mice (2 out of 9 animals). The changes affected the glomeruli, tubules, and interstitial areas of the kidney. There were no vascular changes to the intimal regions of any small, medium, or large sized artery in any of the mice within all groups. The glomeruli showed changes consistent with focal segmental glomerulosclerosis (Fig. 8, C and D) with increased number of glomeruli affected in DOC-salt α-CGRP KO mice compared with the DOC-salt WT mice. Total glomerulosclerosis consisting of total replacement of the glomerulus by fibrous tissue was greater in the DOC-salt α-CGRP KO mice (10 glomeruli overall) compared with the DOC-salt WT mice (2 glomeruli overall). The GSI score was significantly higher in the DOC-salt α-CGRP KO mice (2.2 ± 0.19) compared with the DOC-salt WT mice (1.2 ± 0.18). In the DOC-salt α-CGRP KO mice treated with hydralazine, there were no animals with focal segmental glomerulosclerosis or total glomerulosclerosis; however, there were small amounts of collagen stained by Masson’s trichrome that could be identified within the glomeruli (Fig. 8E). Hydralazine treatment reduced the GSI to 1.1 ± 0.9 and only had lower grading of glomeruli (1 or 2 out of 4) in the scoring system. The tubulointerstitial
changes consisted of multiple areas of fibrosis with chronic inflammation surrounding the tubules primarily made up of lymphocytes, plasma cells, and macrophages. DOC-salt α-CGRP KO mice treated with hydralazine had no interstitial fibrosis; however, chronic inflammation was present. All three groups had dilated tubules with some containing red blood cell casts (Fig. 8F). Regenerative changes were seen in nondilated tubules consisting of apically located nuclei, many binucleated tubular epithelial cells and occasional karyolysis of nuclei. These changes are more prominent in DOC-salt WT mice and DOC-salt α-CGRP KO mice. The tubulointerstitial injury score was higher in the DOC-salt α-CGRP KO mice (2.6 ± 0.5) compared with the DOC-salt WT mice (1.9 ± 1.3) where the DOC-salt α-CGRP KO mice treated with hydralazine was the lowest (1.3 ± 0.8). However, there were no significant differences between any of the groups.

DISCUSSION

The main findings of this study are as follows: 1) hydralazine treatment over the course of the 28-day DOC-salt protocol equalized the BP of the DOC-salt α-CGRP KO mice to the levels seen in the DOC-salt WT mice; 2) kidney dysfunction and oxidative stress were significantly increased in the DOC-salt α-CGRP KO mice compared with DOC-salt WT mice and neither parameter was improved by BP equalization; 3) renal macrophage infiltration, desmin expression, a marker for podocyte damage, and the levels of proinflammatory chemokines and cytokines were significantly higher in the DOC-salt α-CGRP KO mice compared with DOC-salt WT mice and these increases were not attenuated by hydralazine treatment with the exception of IL-6; and 4) BP equalization did, however, result in an improvement in the renal histopathological damage seen in DOC-salt α-CGRP KO mice.

Our strategy to equalize the MAP of a group of DOC-salt α-CGRP KO mice to that of DOC-salt WT mice involved altering the daily dose of hydralazine (in the drinking water). Hydralazine is a direct vasodilator of arteries and arterioles; however, it has some potentially confounding effects. Hydralazine can elicit a reflex sympathetic increase in the heart rate. We did not observe any significant increase in heart rate in the hydralazine-treated group. In addition, there are conflicting reports regarding the effect of hydralazine on oxidative stress (5, 6). However, hydralazine treatment did not reduce urine isoprostane levels. While the BP equalization using daily hydralazine titration was technically difficult, we did achieve our goal of reducing the MAP and systolic BP of the group of DOC-salt α-CGRP KO mice to that of the DOC-salt WT mice (Fig. 1). This same pattern was observed when the nocturnal and diurnal telemetry data were analyzed separately. If anything, the MAP of the hydralazine-treated group tended to be lower and reached statistical significance at four time points (days 9, 15, 20, and 29). The potential significance of this on
injury. Podocytopenia occurs after injury and that ultimately culminates in glomerulosclerosis (1, 17, 21, 32). In a seminal study, ultrastructural alterations of podocytes were demonstrated in DOC-salt rats by electron microscopy and it was reported that podocyte damage, rather than mesangial expansion, triggers the subsequent glomerulosclerosis (13).

Multiple mechanisms underlie DOC-salt hypertension-induced renal damage such as altered renal hemodynamics and paracrine/autocrine or hormonal factors including increased sympathetic nervous system activity, endothelin I levels, angiotensin II levels, and reactive oxygen species (ROS; Refs. 14, 15, 18, 30, 31). All of these factors, particularly increased ROS, contribute to the marked inflammatory response that is likely responsible for much of the tissue damage. Hydralazine treatment of the DOC-salt α-CGRP KO mice did not reduce urinary isoprostane levels, suggesting a direct cellular BP-independent antioxidant role for α-CGRP in this context. In addition, it is well documented that α-CGRP decreases resistance and increases renal perfusion (2, 25, 26, 28). Sensory nerve endings containing CGRP have been localized to the renal vasculature with the afferent arterioles being more sensitive to the dilator effects of CGRP compared with the efferent arterioles. Therefore, the absence of α-CGRP would lead to the loss of the compensatory protective role of this sensory neuropeptide against the increase in renal vascular resistance, resulting in exacerbation of glomerular ischemia and enhanced generation of ROS and subsequent inflammation (30, 31). This possibility would also be independent of systemic BP.

The DOC-salt hypertension-induced inflammatory response in the kidney has been shown to consist of increased extracellular adhesion molecules and chemokines followed by infiltration by monocytes/macrophages and lymphocytes which elaborate additional chemokines as well as pro- and anti-inflammatory cytokines (3, 24, 25, 28). We previously demonstrated that the inflammatory markers ICAM-1, VCAM-1, and MCP-1 were elevated in the kidneys from DOC-salt WT mice compared with their normotensive counterparts (2). In this study, a much greater increase was observed in the DOC-salt α-CGRP KO mice compared with the DOC-salt WT mice. Since these peptides are critical for leukocyte infiltration, in the current study we demonstrated that renal monocyte/macrophage infiltration was significantly higher in the DOC-salt WT

**Fig. 6.** Effects of hydralazine on renal cytokine and chemokine levels. Kidney protein samples from the 5 groups (n = 6/group) of mice were assayed for selected cytokines (A) and chemokines (B). MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α. Values are shown as the means ± SE. *P < 0.001 vs. WT and KO; +P < 0.01 vs. DOC WT.

The interpretation of the histopathological results observed will be discussed below.

The reduction of the MAP by hydralazine treatment in the DOC-salt α-CGRP KO mice did not improve the significant increase in urinary microalbumin excretion and significant decrease in creatinine clearance beyond that observed in the DOC-salt-treated WT mice, indicating that the absence of α-CGRP exacerbates DOC-salt-induced renal dysfunction independent of BP. Our results that demonstrate renal dysfunction in the DOC-salt WT mice in the absence of significant histopathological alterations are consistent with studies from other investigators using the DOC-salt model in C57/BL6 WT mice (20). Ultrastructural changes that lead to microalbuminuria and decreased creatinine clearance include thickening of the glomerular basement membrane, damage to the podocytes leading to foot process fusion, and microvillus transformation of glomerular epithelial cells, which precede the development of lesions that are detectable by light microscopy (20, 30, 31). In both human and animal models of renal damage, increased desmin staining is one of several markers for podocyte damage and is associated with early ultrastructural pathological changes that can only be detected by electron microscopy and significantly elevated proteinuria (17, 21). Our findings are consistent with these results, and more importantly, they are indicative that α-CGRP has a BP-independent protective activity that significantly attenuates podocyte damage, which is a critical early target in the initiation of renal injury. Podocytes and their slit diaphragms play a major role as a size-selective permeability barrier, and podocyte injury is strongly linked to proteinuria. Podocytes typically do not proliferate following

**Fig. 7.** Renal 6-keto-prostaglandin F1α (6-keto-PGF1α) levels are elevated only in the DOC-salt WT mice. Bar graphs show the levels of the prostacyclin breakdown product 6-keto PGF1α in the renal cortex from WT sham (WT), α-CGRP sham (KO), DOC-salt WT (DOC WT), DOC-salt α-CGRP KO (DOC KO), and hydralazine-treated DOC-salt α-CGRP KO (DOC KO HYD) at the 28-day time point. Values are shown as the means ± SE. *P < 0.05 DOC WT vs. all other groups.
mice compared with the sham operated controls. This increase in monocyte/macrophage content was markedly exacerbated in the DOC-salt α-CGRP KO mice, and hydralazine treatment was without effect. Since the immune cells produce additional chemokines and cytokines to modulate the inflammatory response, the expression pattern of the proinflammatory chemokines MCP-1 and MPI-1 was similar to that of monocyte/macrophage infiltration. Thus, with the exception of IL-6, the absence of α-CGRP in the context of DOC-salt-induced hypertension consistently exacerbates renal chemokine production and monocyte/macrophage trafficking to the sites of inflammation in a BP-independent manner. It is unclear why the IL-6 response to hydralazine was different from that if the other proinflammatory chemokines and cytokines were examined.

The inhibitory activity of α-CGRP on the numerous components of the DOC-salt hypertension-induced inflammatory response also involves multiple mechanisms. As described previously, the potent vasodilator activity of α-CGRP enables it to play a compensatory role to attenuate the BP increase in several models of experimental hypertension (7, 23, 27, 28). It also acts directly in the kidney by decreasing renal vascular resistance and increasing the glomerular filtration rate leading to enhanced diuresis and natriuresis. Increased ROS generation is a primary component of hypertension-induced renal damage, including DOC-salt hypertension (2, 30, 31). Oxidative stress leads to activation of transcription factors such as NF-κB. Activated NF-κB induces the expression of inflammatory response genes. Several reports indicate that CGRP can significantly inhibit NF-κB activation (3, 24, 30, 31). CGRP also has been shown to have antioxidant activity through the increased generation of NO that attenuates oxidative stress through the inhibition of reduced nicotinamide-adenine dinucleotide phosphate oxidase (30, 31). Other reports indicate that α-CGRP can inhibit both neutrophil adhesion and platelet aggregation to endothelial cells, thereby ameliorating endothelial dysfunction.

Fig. 8. Sections stained with Masson’s trichrome showing the effects of DOC-salt-induced hypertension on the kidney of WT and α-CGRP KO mice. Glomeruli were histologically unremarkable in sham WT (A) and sham α-CGRP KO mice. B: glomeruli of DOC-salt WT (C) and DOC-salt α-CGRP KO (D) mice showed focal segmental nodular sclerosis (green arrows). DOC-salt α-CGRP KO animals treated with hydralazine (E) showed less build up of collagen within their glomeruli. A representative area of tubulointerstitial changes (F) in a DOC-salt WT mouse shows dilated tubules with RBC casts (blue arrow) and interstitial fibrosis with chronic inflammation. All pictures were taken at ×400 magnification.

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and renal injury (2, 30, 31). CGRP has also been demonstrated to significantly attenuate the inflammatory response to ischemia/reperfusion injury in the liver, kidney, gut, and heart via the stimulation of endothelial cell production of prostacyclin, which is itself a potent cytoprotective anti-inflammatory agent (19). In the present study, we observed that renal prostacyclin generation was markedly enhanced only in the DOC-salt WT mice, which suggests that this may be another mechanism underlying the reno-protective activity of α-CGRP. This finding also indicates that in this context increased prostacyclin generation is dependent on α-CGRP either directly or indirectly, possibly through increased NO production (19).

Interestingly, BP equalization by hydralazine treatment did improve the renal histopathological damage seen in the DOC-salt CGRP KO mice. Numerous studies demonstrate that the histopathological pattern of injury in the kidney is characteristic of direct BP mediated injury (9). In most studies, there is a strong correlation between BP and renal damage although the BP threshold at which renal injury that can be visualized histopathologically may vary between types of hypertension, strain differences, and differences between individual animals (9, 16). We have demonstrated a nonlinear relationship between BP, proteinuria, and kidney damage, as determined by light microscopic histopathology. This is consistent with one of the first reports documenting that mice with a C57/BL6 genetic background were much more resistant to DOC-salt hypertension-induced kidney damage compared with mice with the 129/Sv backgrounds, it was demonstrated that while the BP correlated with glomerulosclerosis, albuminuria did not (10).

In another study, DOC-salt hypertensive rats were treated with the combination of hydrochlorothiazide, reserpine, and hydralazine or the aldosterone antagonist spironolactone (11). The absence of any decrease in BP and proteinuria was attributed to direct mineralocorticoid effects on renal inflammation and fibrosis. Whether a similar mechanism is operative in our model is not known, however, it has been demonstrated that CGRP inhibits the renal activity of the renin/angiotensin/aldosterone system (24, 27, 31). In addition, it has been proposed that salt has direct BP-independent deleterious effects (4). With the use of telemetric recording, WT and α-CGRP KO mice were salt loaded for 4 wk. This treatment increased the MAP in the α-CGRP mice but did not reach statistical significance in the small groups of mice studied. There were also no histopathological changes in the kidneys of either group of mice (unpublished observations, Katki KA, Supowit SC).

In the DOC-salt α-CGRP KO mice where the BP has been equalized to that of the DOC-salt WT mice, there is a dissociation in the temporal relation between enhanced renal oxidative stress, inflammation, podocyte damage, dysfunction, and renal histopathology. Although a threshold relationship between BP and renal damage has been demonstrated, this threshold may be relatively small in terms of an elevation of systemic BP over an extended time. In this present study, the MAP of the hydralazine-treated DOC-salt α-CGRP KO mice tended to be lower than that of the DOC-salt WT mice. Although speculative, this relatively small difference in MAP could result in a lag between the increased oxidative stress, inflammation, and kidney dysfunction and the appearance of lesions that are detectable by light microscopy.

In summary, these studies described herein demonstrate that the impairment of the newly described “effector function” of sensory nerves, mediated by the absence of α-CGRP, significantly exacerbates kidney damage in DOC-salt hypertension. This effect is mediated primarily through a marked increase in oxidative stress and inflammation and involves mechanisms that are both independent and dependent on BP. Therefore, targeting sensory nerve function through the use of CGRP analogs may have therapeutic value in the treatment of hypertension-induced kidney damage.

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