Oxidative Stress

An Ongoing Role of α-Calcitonin Gene–Related Peptide as Part of a Protective Network Against Hypertension, Vascular Hypertrophy, and Oxidative Stress


Abstract—α-Calcitonin gene–related peptide (αCGRP) is a vasodilator, but there is limited knowledge of its long-term cardiovascular protective influence. We hypothesized that αCGRP protects against the onset and development of angiotensin II–induced hypertension and have identified protective mechanisms at the vascular level. Wild-type and αCGRP knockout mice that have similar baseline blood pressure were investigated in the angiotensin II hypertension model for 14 and 28 days. αCGRP knockout mice exhibited enhanced hypertension and aortic hypertrophy. αCGRP gene expression was increased in dorsal root ganglia and at the conduit and resistance vessel level of wild-type mice at both time points. βCGRP gene expression was also observed and shown to be linked to plasma levels of CGRP. Mesenteric artery contractile and relaxant responses in vitro and endothelial NO synthase expression were similar in all groups. The aorta exhibited vascular hypertrophy, increased collagen formation, and oxidant stress markers in response to angiotensin II, with highest effects observed in αCGRP knockout mice. Gene and protein expression of endothelial NO synthase was lacking in the aortae after angiotensin II treatment, especially in αCGRP knockout mice. These results demonstrate the ongoing upregulation of αCGRP at the levels of both conduit and resistance vessels in vascular tissue in a model of hypertension and the direct association of this with protection against aortic vascular hypertrophy and fibrosis. This upregulation is maintained at a time when expression of aortic endothelial NO synthase and antioxidant defense genes have subsided, in keeping with the concept that the protective influence of αCGRP in hypertension may have been previously underestimated. (Hypertension. 2014;63:1056-1062.) ○ Online Data Supplement

Key Words: angiotensin II ■ calcitonin gene-related peptide ■ hypertension ■ hypertrophy ■ mice ■ nitric oxide ■ oxidative stress

α-Calcitonin gene–related peptide (αCGRP) is a potent vasodilator1 and a hypotensive peptide. It is primarily localized to the sensory nervous system, with a perivascular innervation and considered to be the major cardiovascular form, as compared with the structurally similar βCGRP. CGRP acts via a G-protein–coupled receptor (calcitonin-like receptor) when dimerized with a single transmembrane-spanning receptor activity–modifying protein RAMP12 signaling via cAMP and other pathways.3,4

CGRP does not play a primary role in the regulation of basal blood pressure (BP) in normal individuals5,6 but is suggested to have protective properties, in cardiovascular disease,7,8 including attenuation of vascular smooth muscle proliferation,9 hyperplasia,10,11 and stimulation of endothelial cell proliferation12 and endothelial progenitor cells.13 Evidence indicates the importance of CGRP in aggressive models of rodent hypertension that are centered on the kidney.14,15 By comparison, there is little evidence of detailed analysis involving the ongoing influence of endogenous CGRP on hypertensive mechanisms and vascular remodeling, especially with regard to NO and oxidative stress pathways.

Sensory nerve–derived CGRP release is stimulated by mechanisms that include angiotensin II (AngII) and sympathetic nerve reflexes,1,4 baroreflex sensitivity,16 and sensory nerve activators.17,18 We have investigated the AngII hypertension model in wild-type (WT) and αCGRP knockout (αCGRPKO) mice that have similar resting BP. We hypothesized that αCGRP is protective against the onset and development of hypertension, and the aim was to identify mechanisms by which αCGRP is protective in this model. The novel findings show
that αCGRP KO mice demonstrate enhanced hypertension ≤28 days, with evidence of continued increasing αCGRP gene and protein expression in WT mice. The results identify αCGRP as a critical mediator in the protection against the onset and development of AngII-induced hypertension by enhancing the ability of the vasculature to protect against early mechanisms involved in vascular remodeling and oxidative stress.

Materials and Methods
An expanded Methods section is available in the online-only Data Supplement.

Mouse Models
C57BL/6 WT and αCGRP KO mice were characterized previously. Mice were divided into mixed sex groups, and either vehicle (saline) or AngII was infused (1.1 mg/kg per day for 14 days or 0.9 mg/kg per day for 28 days). BP was recorded in conscious mice by tail-cuff technique, and samples were analyzed ex vivo. All experiments were performed according to the Animals (Scientific Procedures) Act, 1986, United Kingdom and the institutional ethical review committee.

Statistical Analysis
Results are expressed as means±SEM and analyzed by 2-way ANOVA and Bonferroni post hoc test, unless stated. Significance was accepted as P<0.05.

Results
CGRP Deletion Enhances AngII-Induced Hypertension
WT and αCGRP KO mice exhibit a similar baseline BP (Figure S1A–S1C in the online-only Data Supplement). By comparison, infusion of WT mice with AngII (WT-AngII) led to a significant hypertensive state, characterized by increased BP (Figure 1A–1C) and heart:body weight ratio (Figure S1D), compared with vehicle-treated mice. αCGRP mice treated with AngII (αCGRP KO-AngII), showed an enhanced BP, compared with WT mice (Figure 1A–1C). A further sustained study for 28 days demonstrated a similar profile with αCGRP KO-AngII mice showing a significantly enhanced hypertension compared with AngII-WT mice (Figure 1A–1C).

Effect of AngII on CGRP and Receptor Expression
At 28 days, there was an increase in the receptor components calcitonin-like receptor and RAMP1 mRNA in the aortas of WT-AngII 28 but not αCGRP KO-AngII mice (Figure S2A and S2B). αCGRP mRNA expression was increased in both conduit (aorta, Figure 2A) and resistance vessels (mesenteric resistance vessels [MRVs], Figure 2C) and dorsal root ganglia (DRG; Figure S3A) of WT-AngII treated, compared with vehicle-treated mice. These data indicate the sensory nerves as a major source of CGRP. Surprisingly, plasma levels of CGRP were raised in αCGRP KO-AngII as well as WT-AngII mice (Figure 2E). Further investigation revealed an increase in βCGRP mRNA (Figures 2B and 2D and S3B), indicative of an additional source of circulating CGRP.

Vascular Reactivity of the MRVs
The prominent role of the resistance vessels in BP regulation led us to investigate the reactivity of mesenteric arteries. Results indicated a normally functioning mesenteric vasculature, in terms of relaxation to CGRP (direct acting), carbachol (endothelium dependent), and constriction to phenylephrine (α1-adrenergic receptor agonist) and U46619 (the thromboxane A2 receptor agonist; Figure S4A–S4D).

Hypertrophy and Aorta
The thoracic aortae of WT-AngII mice were analyzed by Masson trichrome (Figure 3A). They showed both increased aortic wall width (Figure 3B) and collagen area (blue staining surrounding aortic wall, Figure 3C). Hypertrophy was enhanced at day 14 in the αCGRP KO-AngII when compared with WT mice (Figure 3A–3C). Increased vascular hypertrophy was also observed at 28 days, but was more profound in the aortic wall as opposed to the adventitial layer. Collagen mRNA expression was also increased (Figure 3D) in WT-AngII at 14 days and especially in αCGRP KO-AngII mice at 14 and 28 days.

Endothelial NO Synthase and Endothelin Expression
The finding that only the aorta exhibited vascular abnormalities led us to study endothelial NO synthase (eNOS) expression. The eNOS mRNA expression was similar in MRV samples, regardless of treatment (Figure 4A). By comparison, there was a loss of eNOS mRNA expression in aortae after 14 days in WT-AngII which was significant in αCGRP KO-AngII mice and a substantial loss of eNOS in both WT-AngII and KO-AngII

Figure 1. Effect of angiotensin II (AngII) on blood pressure in wild-type (WT) and α-calcitonin gene–related peptide knockout (KO) mice treated with either vehicle or AngII for 14/28 days, measured by tail-cuff plethysmography (n=3–9). A, Systolic blood pressure. B, Diastolic blood pressure. C, Mean arterial pressure. *P<0.05, **P<0.01, and ***P<0.001 vs respective vehicle-treated control. #P<0.05 vs respective WT-AngII mice.
mice aortae by 28 days (Figure 4B). There was significantly less eNOS protein in αCGRPKO-AngII aortic compared with WT-AngII aortic samples at 28 days (Figure 4C and 4D). An increased expression of endothelin was also investigated for, but no significant differences were found. Possibly endothelin becomes important at later time points (Figure S5A and S5B).

NADPH Oxidase 2 and 4 and Markers of Reactive Oxidative Stress in the Aorta

NADPH oxidase 2 (Nox2) and Nox4 levels were increased at 14 days (Figures S6 and S7). In the case of Nox2, the mRNA expression was further enhanced in αCGRPKO-AngII mice (Figure S6A), but protein expression was similar in both (Figure S6B). Specifically, a striking upregulation in Nox4 mRNA (Figure S7A) and protein expression (Figure S7B) is shown in αCGRPKO-AngII mice at 14 days. Increased total Nox4 immunoreactivity at 14 days was primarily localized with collagen (Figure S7C–S7D) in keeping with a role in vascular remodeling/fibrogenesis. However, by 28 days, mRNA expression of both NADPH oxidases was blunted, suggesting a transient effect on Nox2 and Nox4 expression during the early phase in the AngII-mediated hypertension.

αCGRP deletion was associated with increased mRNA expression of the endogenous markers of oxidative stress heme oxygenase-1, glutathione peroxidase-1 (GPX-1), and superoxide dismutase-1 (Figure 5A–5C) at 14 days, suggesting an ongoing presence of these enzymes in attempting to reduce oxidative stress. By comparison, at 28 days, levels were higher in WT-AngII mice, but, by, heme oxygenase-1 and GPX-1 increased expression had been lost in αCGRPKO-AngII mice. Concomitantly, there was evidence indicative of increased reactive oxidative stress (ROS) generation in αCGRPKO-AngII mice, because levels of nitrotyrosine, a marker of nitrated proteins, and 4-hydroxynonenal, a marker of lipid peroxidation (Figure 5D–5F), were enhanced.

Discussion

A clear involvement of αCGRP in protecting against the onset and ongoing AngII-induced hypertension and associated pathophysiology is shown. First, an increased mRNA expression of αCGRP in DRG, aorta, and mesenteric vessels and of the CGRP receptor components at 28 days in the aorta indicates the increased activity of this pathway. Second, αCGRP deletion leads to enhanced hypertension, vascular hypertrophy, and a premature loss of eNOS message and protein in the αCGRPKO aorta, at a time when the mesenteric eNOS and vasoactive responses remain unaltered. Third, αCGRP deletion is associated with enhanced oxidative stress and a loss of...
endogenous antioxidant expression in the conduit aorta. This implies that αCGRP is part of a protective network, protecting the aorta of the hypertensive mouse against vascular remodeling and oxidative stress.

We show here that the untreated selective αCGRPKO mouse has normal BP, in keeping with the concept that CGRP is not involved in the physiological regulation of normal BP. By comparison, the most studied αCGRPKO mice also have the calcitonin gene deleted and are spontaneously hypertensive.20 However, CGRP release has been described as a key compensatory mechanism against elevating BP.21 Thus, the BP results observed here at 14 days are expected. Importantly, hypertension remains exacerbated in αCGRPKO-AngII at day 28 compared with WT-AngII, suggesting a sustained protective role of αCGRP.

The hypertension was accompanied by increased αCGRP gene expression in WT DRG, the major site of sensory nerve–derived CGRP synthesis, as well as in the conduit (aorta) and resistance (mesenteric) vasculature ≤28 days. Thus, a sustained protective influence of αCGRP is in keeping with its vasodilator properties. Indeed, the acute administration of exogenous αCGRP is known to decrease BP in the spontaneously hypertensive rat22 also, over 6 days in the AngII-hypertensive rat.23 However, an early study of AngII-induced hypertension in the rat did not show an upregulation of DRG CGRP expression.24

Plasma CGRP levels were raised in hypertensive WT and αCGRPKO mice in the present study. These data support Portaluppi et al25 who reported a parallel increase in plasma CGRP levels, with increases in BP after AngII infusion in humans, in contrast to others who have shown decreases in plasma CGRP in essential hypertension.3 The similarity in CGRP structures (>90%) precludes generation of a selective αCGRP antibody. Here, βCGRP gene expression is also observed. The lesser-researched βCGRP possesses similar biological activity4 and may contribute additionally to the protective effect of αCGRP observed here, as well as to plasma CGRP levels in αCGRPKO-AngII mice. It has recently been identified in the adventitia of rat mesenteric branch arteries.26 The decreased plasma levels observed in some human hypertension studies may be attributable to lack of β rather than αCGRP, leading a misleading concept where decreased levels of plasma CGRP are falsely correlated with a lack of involvement of αCGRP.

An increase in vascular CGRP receptor gene expression (both calcitonin-like receptor and RAMP1) in acute AngII rat studies (10 days) was suggested to further amplify responses to CGRP,21 although our study is the first to show upregulation chronically (28 days), to our knowledge. The use of RAMP1 transgenic mice revealed that CGRP has an early protective effect at the level of the baroreceptors and cerebral vasculature in a similar AngII hypertension model.16,27 Our present results indicate a much wider potential of CGRP and its receptors to protect against the onset and maintenance of AngII-induced hypertension.

Analysis of MRVs after 28 days revealed similar vascular reactivity in all treatments with no loss of

Figure 3. Effect of angiotensin II (AngII) on aortic remodeling in wild-type (WT) and α-calcitonin gene–related peptide knockout (αCGRPKO) mice treated with either vehicle (saline) or AngII for 14/28 days. A, Representative images of Masson trichrome stained aortic sections from WT and αCGRPKO mice after vehicle or AngII treatment at both 14- and 28-day time points. Quantification of (B) smooth muscle cell (SMC) area and (C) collagen area (n=6–10). D, Aortic mRNA expression of collagen III measured by quantitative reverse transcription polymerase chain reaction (n=5–6). *P<0.05, **P<0.01, and ***P<0.001 vs respective vehicle-treated control, #P<0.05, and ##P<0.01 vs respective WT-AngII mice.
endothelium-dependent relaxant activity or of MRV mRNA eNOS expression. By comparison, eNOS gene expression was reduced in aortae of WT-AngII and significantly more so in the αCGRPKO-AngII mice, at 14 days and maintained at 28 days, with also significant eNOS protein loss, in keeping with αCGRP protecting against loss of eNOS, an early marker of endothelial dysfunction in the aorta. Interestingly, the lack of loss of eNOS expression in the mesentery of KO-AngII mice correlates with increased mRNA expression of βCGRP, potentially implicating this β form as a protective factor. The sustained increase in hypertension and of aortic vascular hypertrophy in the αCGRPKO mouse implies that αCGRP can, by comparison, maintain a protective influence on the cardiovascular system, in the presence of ongoing AngII stress. The concept that αCGRP may continue to act in a protective manner in aorta, when eNOS expression is diminishing, is to our knowledge, new. A greater onset of aortic remodeling was observed in αCGRPKO-AngII compared with WT-AngII mice, in terms of collagen deposition and vascular smooth muscle cell hypertrophy, in keeping with reports that CGRP inhibits proliferation of vascular smooth muscle cells via mechanisms that include cAMP elevation or inhibition of the extracellular signal-regulated kinases1/2 pathway.

AngII mediates ROS generation, provoking oxidative stress–mediated vascular hypertrophy in this AngII hypertension model as well as Nox2 expression. Our results show an increase in Nox2 and Nox4 in αCGRPKO-AngII compared with WT-AngII mice with enhanced expression of Nox2 throughout the 28 days. Surprisingly, a substantial upregulation of Nox4 gene and protein expression in the adventitia of the aorta of αCGRPKO-AngII by day 14 was observed. Previous studies have only shown modest protein levels of Nox4 in the aorta of hypertensive rodents. Nox4 is considered to contribute to NAD(P)H-derived ROS and inhibit migration in activated adventitial fibroblasts. However, because the enhanced Nox4 gene expression was diminished in AngII-treated WT and KO mice by 28 days, this may suggest a similar relatively early loss of modulatory influence of Nox4 to that of eNOS and other endogenous antioxidants.

Evidence of enhanced oxidative stress and increased activity of the endogenous antioxidant system (heme oxygenase-1, superoxide dismutase-1, and GPX-1) is observed in αCGRPKO-AngII mice at 14 days, in keeping with previous AngII-murine models, thought to counteract loss of NO bioavailability and ROS-mediated vascular smooth muscle hypertrophy and remodeling. These enzymes play a crucial role in the scavenging of superoxide radicals, which are produced in response to elevated BP, AngII upregulation, and a reduction in NO bioavailability. Mouse models of GPX-1 deletion have shown exacerbated hypertrophy after AngII infusion, providing evidence of protection and that heme oxygenase-1 induction counteracts hypertension. The compromised antioxidant gene expression in αCGRPKOs at day 28 correlates with enhanced disease progression. The concomitant hypertrophy in αCGRPKOs and loss of increased GPX-1 gene expression at 28 days is in keeping with studies using GPX-1 KO mice which revealed protection against AngII-induced artery functional and cardiac remodeling changes.

We provide novel evidence linking αCGRP with vascular protection against enhanced oxidative stress in a model of hypertension, consistent with evidence from cardiac models and an atherosclerosis model. We further provide evidence of enhanced ROS generation in αCGRPKO-AngII mice at day 28, as shown recently in a wire-induced vascular injury model.

We conclude that mice lacking αCGRP are predisposed to excessive hypertension that persists for ≥28 days after AngII treatment. We think that the total involvement of CGRP has been previously underestimated, at least in murine models. We demonstrate evidence for β, in addition to αCGRP gene expression. We have also shown that αCGRP is protective at
the aortic level preventing loss of eNOS, and ROS production through maintaining scavenging by antioxidant enzymes at a time when eNOS expression and mesenteric vascular resistance remains normal. Moreover, the results provide support for the concept that αCGRP agonists have potential as novel antihypertensive agents.

**Perspectives**

CGRP has protective effects in cardiovascular disease, but the mechanisms are unclear. Here, we support previous reports that αCGRP is protective in hypertension and provide evidence that αCGRPKO mice are more susceptible to the development and severity of AngII-induced hypertension for ≤28 days. There was increased DRG and vascular αCGRP expression in WT mice, implying the upregulation of the αCGRP sensory axis and a direct link of this to the protected cardiovascular phenotype observed in WT mice. Surprisingly, we provide evidence that βCGRP is also upregulated, with a likely contribution to the plasma CGRP levels that were measured in αCGRPKO mice. The adverse effects in αCGRPKO mice included aortic remodeling, a more rapid loss of aortic eNOS, and loss of the antioxidant defense system, and these findings provide the novel mechanistic aspect to this study. This study provides evidence that αCGRP can protect the aorta at a key time when remodeling is occurring, the protective role of eNOS is diminishing, and oxidative stress is primed. These combined results indicate that the protective role of CGRP in the onset and maintenance of the aortic vasculature may have been previously underestimated. They provide support for the concept that a stable CGRP agonist may have therapeutic potential.

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**Disclosures**

None.

**References**


29. What Is New?

- Angiotensin II–induced hypertension is exacerbated in mice lacking the αCGRP gene both at 14 and 28 days.

**Novelty and Significance**

**What Is New?**

- Angiotensin II–induced hypertension is enhanced in α-calcitonin gene-related peptide (αCGRP) knockout mice for ≥28 days.

- αCGRP is upregulated in wild-type mice that show reduced aortic loss of endothelial NO synthase and of the antioxidant defense system.

**What Is Relevant?**

- The protective ability of αCGRP may have previously been underestimated because it is ongoing at a time when that to NO is curtailed.

- The results provide enthusiasm for the concept that stable αCGRP agonists may be potential therapeutic targets for antihypertensive therapies.

**Summary**

Angiotensin II–induced hypertension is exacerbated in mice lacking the αCGRP gene both at 14 and 28 days.
An Ongoing Role of α-Calcitonin Gene–Related Peptide as Part of a Protective Network Against Hypertension, Vascular Hypertrophy, and Oxidative Stress


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AN ONGOING ROLE OF CGRP AS PART OF A PROTECTIVE NETWORK AGAINST HYPERTENSION, VASCULAR HYPERTROPHY AND OXIDATIVE STRESS

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Short Title: CGRP and Angiotensin II-induced hypertension

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**Detailed Materials and Methods**

**Mouse Model**
Wild-type (WT) and alpha CGRP knockout (αCGRPKO) C57BL/6 mice were bred in-house. All were maintained on a normal diet, with free access to food and water, in a climatically controlled environment, on a 12 hour light (7am-7pm) / dark (7pm-7am) cycle. αCGRPKO mice were age and sex matched with their respective WT littermates. All experiments were carried out in accordance with the Animals (Scientific Procedures) Act 1986. Both genders of mice (18-30g) were used in these experiments at the age of 2 months.

**Generation of αCGRP KO mice**
αCGRPKO and WT mice were a gift from Dr. A.M. Salmon, Institute Pasteur, Paris, France. αCGRPKO mice were created by disruption of exon 5 (specific to αCGRP) of the calcitonin/αCGRP gene by a cassette containing lacZ/CMV/neomycin resistance genes. They were bred from pairs of both heterozygous mice and knockout mice, generating an equal mixture of heterozygous and homozygous knockout offspring. This strain displays normal growth and behavioural characteristics, and have been used previously. More recently, our group have used these mice to measure vasodilation responses to mustard oil. Confirmation of the genotype of the mice was carried out using end point PCR to identify the presence (wildtypes/heterozygotes) or absence (knockouts) of the functional αCGRP gene (Figure S8) using a small ear sample taken under 2% isoflurane and 2% O₂ (Abbott Laboratories, Kent, UK) anaesthesia. Isolation of genomic DNA was done using a commercially available kit (ExtractRED-N-Amp tissue PCR kit, Sigma, UK) and the resulting isolated genomic DNA was then amplified for the gene of interest in a thermocycler using the following primers for αCGRP: Primer A: 5’CCCCTAATGGCCTTGTGATTG’3; Primer B: 5’ACCTCCTGATCTGCTCAGCAG’3; Primer D: 5’GATGGGCGCATCGTAACCCGT’3. The amplified signal was then visualised by gel electrophoresis and DNA bands were visualised under ultraviolet (UV) light.

**Angiotensin II Murine Model**
Alzet osmotic minipumps (Charles River UK), with either a 14 or 28 day capacity, average volume of 100µl, and an infusion rate of 0.23µl/hr for 14 days or 0.10µl/hr for 28 days, were filled with either saline (vehicle) or angiotensin II (Sigma, UK) at a concentration adjusted to the weight of the mouse. This was to allow for a continuous dose of AngII at 1.1mg/kg/day for 14 days (as previously demonstrated) or 0.9mg/kg/day for 28 days. Pumps were implanted subcutaneously in the mid-scapular region under isoflurane anaesthesia and pain relief was administered intramuscular with Buprenorphine (50µg/kg, Vetergesic, Alstoe animal health). Insertion wounds were sutured with absorbable sutures (4.0, Ethicon, Johnson and Johnson). Mice were then housed singly and monitored daily. The animals were divided into four groups: WT vehicle (saline), αCGRPKO vehicle (saline), WT-AngII and αCGRPKO-Ang II. BP was then recorded every day by tail cuff plethysmography until day 14 or 28 when the experiment was terminated. This was initiated by anaesthetising the animal with isoflurane until a deep surgical level was maintained, and then blood was collected via the left ventricle of the heart by
cardiac puncture to obtain plasma. The animal was then culled via cervical dislocation of the neck and organs were then harvested for post-analysis.

**Measurement of BP via Tail-cuff Plethysmography**

BP was measured by tail cuff plethysmography, using the CODA 8 non-invasive BP acquisition technique system for mice (Kent Scientific, Torrington, CT, USA). The mean of at least five BP measurements were taken per mouse each day. All measurements were taken at a thermoneutral ambient room temperature of 25-27°C. Mice were also warmed on a heating pad for 10 minutes prior and during the BP recordings. Mice were trained for at least 14 consecutive days prior to taking the BP measurements in order to reduce stress-induced hypertension caused by restraint. Measurements were then taken prior to the minipump implantation (day 0) and every day until the animals were terminated (day 14/28).

**Total plasma CGRP (α and β isoforms)**

Circulating plasma CGRP levels were measured using a Rat/Mouse ELISA kit from Phoenix Pharmaceuticals Inc, USA according to manufacturer's instructions. Plasma peptides were extracted using SEP-COLUMN’s (Phoenix Pharmaceuticals Inc, USA, procedure carried out in accordance to manufacturer's instructions) prior to the ELISA to ensure samples were concentrated and purified. Results are expressed as pg/ml and normalised to plasma protein levels, measured using a Bio-Rad DC™ Protein assay (BioRad, UK). Cross-reactivity of this assay occurs with all CGRP forms (78.6% with CGRP II rat and 20.1% human) and to a certain degree with CGRP from other species (100% with rat and 15.1% with human). However it is not cross-reactive with closely related peptides such as calcitonin (0%) and amylin (<0.01%).

**Wire Myography**

The mesenteric bed was excised from the animal and first-order branches of the superior mesenteric artery (~200 µm in diameter) were dissected out in ice-cold Krebs. Vascular reactivity of the mesenteric arteries was characterized with isometric tension studies using wire myography as previously described. Briefly, segments of isolated mesenteric arteries (~2 mm) were mounted on two 25 µm stainless steel wires connected to a force transducer and a micrometer in an organ bath containing Krebs solution aerated with 95% O₂/5% CO₂ (pH = 7.4, temperature = 37°C) of a small vessel myograph (Danish Myo Technologies, Aarhus, Denmark). Following 60 minutes of equilibration at 37 °C, vessel segments were stretched to their optimal internal circumference IC₁ = 0.9×IC₁₀₀, where IC₁₀₀ is an estimate of the internal circumference of the vessel under a passive transmural pressure of 100 mmHg (13.3 kPa), in order to obtain optimal conditions for active tension development. Subsequently, the vessels were contracted repeatedly with 80 mM K⁺ Krebs with the following composition (in mM): NaCl, 38; KCl, 80; CaCl₂, 1.8; NaHCO₃, 24; Mg₂SO₄, 1; NaH₂PO₄, 0.5; Glucose, 5.6 with pH adjusted to 7.4 with 95% O₂ and 5% CO₂ until reproducible wall tensions were recorded. Cumulative concentration-response curves with phenylephrine, U46619, carbamoylcholine chloride (carbachol), and αCGRP were made. Dilatory responses were characterized in vessels precontracted with EC₈₀ (M) of U46619. Endothelial function in the arterial segments was assessed by the relaxation mediated by 10 µM carbachol following a steady precontraction tone induced by 1-100 nM U46619. A threshold of ≥ 60% relaxation with 10 µM carbachol was used for endothelial function. The mean relaxation achieved with 10
µM carbachol was 85% ± 5% (WT Veh), 94% ± 7% (WT AngII), 80% ± 5% (αCGRP KO Veh), and 76% ± 3% (αCGRP KO AngII). These results indicate that the vessel preparations contain a sufficient amount of endothelial cells to perform our experiments.

All drugs were obtained from Sigma-Aldrich (St Louis, MO, U.S.A.), except U46619 (Enzo Life Sciences Inc., NY, USA) and αCGRP (Bachem, Weil am Rhein, Germany), and dissolved in distilled H2O except for U46619 which was dissolved in ethanol. Contractile responses of arterial segments to U46619 and phenylephrine are expressed as percentage of the response induced by 80 mM K+ Krebs solution while vasodilatory responses to carbachol and αCGRP are expressed as percentage of the steady-state preconstriction response to 1-100 nM U46619.

**Aortic Histology**

Aortic sections (5µm) were analysed and quantified for measurements of vascular hypertrophy using Masson’s Trichrome staining as described previously.4, 6

**Nox4 immunohistochemistry in aortic sections**

The thoracic aorta was dissected and fixed in 10% formaldehyde before being processed into paraffin blocks. Serial aortic sections cross sections (7µm) were cut using a microtome. Nox4 (1:500 raised in rabbit, gift from Prof Ajay Shah, Kings College London) expression was assessed by immunohistochemistry using the Envision HRP kit, Dako, UK in accordance to manufacturer’s instructions. Nox4 positive staining was measured by densitometry using imaging programs reported previously.6 Measurements for dark brown staining were considered as positive staining and collected. Results are expressed as % of positive staining relative to total aorta area. Densitometry was calculated on a mean of 10 measurements per mouse, and was further divided into 3 layers; endothelial, smooth muscle cell and collagen/adventitia. This was to allow for localisation of the protein to be determined. All histology samples were analysed blind and referred to by an identifying reference number only.

**Measurement of Gene Expression using Real-Time Polymerase Chain Reaction (RT-PCR)**

Small (10-30mg) tissue samples (aorta, mesenteric resistance vessels and dorsal root ganglia) were excised from the animal and immersed in RNAlater (Ambion) for at least 24 hours prior to extraction. Total RNA was extracted from the tissue using the Qiagen RNeasy Microarray spin column kits (Qiagen) according to manufacturer’s instructions. The total RNA was then eluted in nuclease-free water and RNA concentration and purity was measured on a Nanodrop spectrophotometer to determine 280/260 and 260/230 ratios. A ratio of ~1.8-2.0 was deemed suitable for further analysis. A randomised selection of samples from each batch were analysed for RNA degradation using the Agilent 2100 Bioanalyzer (Applied Biosystems) in accordance to manufacturer’s instructions. RNA was re-extracted or omitted from the study if samples from these batches had a RNA Integrity Number (RIN) of below 6. RNA (500ng) was then reverse transcribed into cDNA using the High Capacity RNA-to-cDNA Kit from Applied Biosystems, as per manufacturer’s instructions. RT was run using a thermal cycler (Applied Biosystems) which was preset according to the manufacturer guidelines. Negative RT samples were carried out as a control to exclude possible contamination of genomic DNA. cDNA was then diluted 1:5 in nuclease-free water for RT-qPCR. Real-time PCR was performed using
a SYBRGreen based PCR mix (Sensi-Mix®, SYBR-green no ROX, Bioline) and primers from the specific gene of interest. A list of primers used is shown in Table S1 and were all obtained from Sigma, UK. Primer sequences were confirmed for specificity and correct product size using Primer Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and amplified PCR products from these sequences were confirmed by running end point PCR gels. All primers used were intron-spanning. qPCR was run using the Corbett Rotorgene 6000 using the following cycle; hold for 10mins at 95°C, cycling for 45 cycles- 10 sec at 95°C, 15 sec at 57°C, 5 sec at 72°C and finally melt 68-90°C. Product specificity of the PCR reaction was assessed by performing a melting point analysis after each run, and efficiency of the reaction was also assessed, with results not being accepted if the efficiency was not >90%. Copy numbers of the genes measured in samples were derived from a standard curve. Data were then plotted against this standard curve using the Rotorgene 6000 series software and expressed as copies/µl of cDNA. In order to compare gene expression between samples, it is necessary to normalise the qPCR data to account for variations in starting material, total or mRNA extraction and reverse transcription efficiency. Normalisation in this case was done running a number of endogenous housekeeping reference genes and then using the three most stable genes which were hypoxanthine guanine phosphoribosyl transferase (HPRT-1), succinate dehydrogenase (SDHA) and phospholipase A 2 (PLA 2). To calculate the geometric mean of the reference genes we used the GeNorm version 3.4 software. The raw data were then normalised by dividing the copies/µl of the gene of interest by a normalisation factor derived by GeNorm which then gives us our final expression value. Statistical analysis was carried out on normalised copies/µl before plotting graphs as an expression of fold change compared to vehicle. The data were expressed this way due to the two studies (14 and 28 days) being run at different times, a factor which was out of our control. This then minimises any experimental variation between the two studies and we can compare trends between the two time-points. Statistics were only carried out within each of the two studies, no statistics were done comparing 14 days against 28 days as this is not viable due to the qPCR being run on different occasions.

**Western Blotting**

Aortae were removed and snap-frozen prior to homogenization in lysis buffer by TissueLyser II (Qiagen, UK). After centrifuging supernatants were collected and a Bio-Rad DC™ Protein assay (BioRad, UK) was performed. 50µg of protein per sample was prepared in sample buffer (90% bromophenol blue: 10% 2-mercaptoethanol) in 1:0.1 of sample: sample buffer dilution factor and were heated at 95°C for 3 minutes. SDS-PAGE was performed on samples in 7% or 12% polyacrylamide gels with 5% stacking gel, run in running buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3) using the Mini-PROTEAN® Tetra cell apparatus (Bio-Rad, UK). The proteins were blotted onto polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, USA) by a semi-dry blotting apparatus (Scie-Plas, UK) in transfer (Towbin) buffer (25mM Tris, 192mM glycine pH 8.3, 20% methanol), before non-specific binding on the membrane was blocked by blocking solution containing: 5% (wt/vol) dried skimmed milk made up in phosphate-buffered saline (PBS) pH 7.3 and 0.1% Tween®-20 (PBST) (Fisher Scientific, UK). The membrane was probed with 1° antibody incubation overnight at 4°C, followed by incubation with 2° antibody. The protein expression was determined by enhanced chemiluminescence (Luminata™ Classico Western HRP Substrate, Millipore, UK).
Images were captured in a Gel Documentation System (G-Box, Syngene Ingenius Bioimaging) and densitometric analyses were performed using the ImageJ Software (NIH, USA).

Primary antibodies used were: anti-NOX4 (1:500) and anti-NOX2 (1:500), both were gifts kindly received from Prof. Ajay Shah, King’s College London; anti-eNOS (1:500); NOS3 (C-20, sc-654), rabbit polyclonal IgG, Santa Cruz Biotechnology); anti-4-hydroxynonenal (1:2000; Anti-4-hydroxynonenal Antibody, rabbit polyclonal antibody (ab46545), Abcam); anti-3-nitrotyrosine (1:500; mouse monoclonal antibody nitrotyrosine [39B6] (ab61392), Abcam); anti-β-actin (1:2000; monoclonal anti–β-actin antibody produced in mouse (clone AC-15 purified immunoglobulin) (A1978-200UL), Sigma).

**Statistical Analysis**

Results are shown as mean ± standard error of the mean (sem) or fold change ± sem, and evaluated by ANOVA + Bonferroni’s post hoc test, using Graph Pad Prism (San Diego California, USA [www.graphpad.com](http://www.graphpad.com)). A significant difference was accepted when p<0.05.
References


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**Table S1.** List of gene primer sequences and amplicon size (base pairs) used for RT-qPCR studies.
Figure S1. Basal systolic (A), diastolic (B) and mean arterial pressure (C) measurements over 14 days in naive WT and αCGRP-KO mice at 2 months of age, measured by tail-cuff plethysmography (n=8). (D) End point body and organ weights of WT and αCGRP-KO mice treated with either vehicle (saline) or AngII for 14 days (n=9-10). **P<0.01 vs respective vehicle treated control and #P<0.05 vs AngII treated WT.
Figure S2. Effect of AngII for 14/28 days on CGRP receptor component mRNA expression in the aortae of WT and αCGRP KO mice, measured by RT-qPCR (n=3-7). (A) Calcitonin like receptor (CLR), (B) Receptor activity modifying protein-1 (RAMP1). *P<0.05 and ***P<0.001 vs respective vehicle treated controls.
Figure S3. Effect of AngII for 14/28 days on both α and βCGRP mRNA expression in the DRG of WT and αCGRP KO mice, measured by RT-qPCR (n=3-7). (A) αCGRP and (B) βCGRP. *P<0.05 and ***P<0.001 vs respective vehicle treated controls. ####P<0.001 vs respective AngII treated WT mice.
Figure S4. Concentration-response relationships in endothelium-intact mesenteric arteries from WT and αCGRP KO mice infused for 28 days with either vehicle (saline) or AngII to (A) Phenylephrine ($10^{-9}$-$10^{-5}$ M), (B) U46619 ($10^{-11}$-$10^{-7}$ M), (C) Carbachol ($10^{-9}$-$3x10^{-5}$ M), and (D) CGRP ($10^{-11}$-$10^{-8}$ M). n=5-6. Relative responses are given as percentage of the vessel response to 80 mM K⁺ Krebs solution and normalized responses as percentage of precontraction tone.
Figure S5. Effect of AngII on ET-1 protein and mRNA expression in WT and αCGRP KO mice treated with either vehicle (saline) or AngII for 14/28 days. (A) Circulating plasma protein ET-1 levels, measured by ELISA at day 14 (n=4) and (B) mRNA expression, measured by RT-qPCR for aortic ET-1 at days 14/28 (n=3-7).
Figure S6. Effect of AngII on Nox2 mRNA and protein expression in WT and αCGRP KO mice treated with either vehicle (saline) or AngII for 14/28 days. (A) mRNA expression, measured by RT-qPCR for aortic Nox2 at days 14/28 (n=3-7) and (B) protein expression measured by Western Blotting for aortic Nox2 at day 14 (n=3). **P<0.01 vs respective vehicle treated control.
Figure S7. Effect of AngII on Nox4 mRNA and protein expression in WT and αCGRPKO mice treated with either vehicle (saline) or AngII for 14/28 days. (A) mRNA expression, measured by RT-qPCR for aortic Nox4 at days 14/28 (n=3-7) and (B) protein expression measured by Western Blotting for aortic Nox4 at day 14 (n=3). Nox4-positive staining was localised by IHC in the adventitia of the aorta, as illustrated in representative images from days 14/28 (D) and quantified ((C), n=4). *P<0.05 and ***P<0.001 vs respective vehicle treated control, ###P<0.001 vs respective AngII treated WT mice.
Figure S8. Typical gel electrophoresis results showing the products of PCR amplification of the αCGRP gene using genomic DNA from WT and αCGRPKO mice.