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A Novel α-Calcitonin Gene-Related Peptide Analogue Protects Against End-Organ Damage in Experimental Hypertension, Cardiac Hypertrophy, and Heart Failure

Editorial, see p 384

BACKGROUND: Research into the therapeutic potential of α-calcitonin gene–related peptide (α-CGRP) has been limited because of its peptide nature and short half-life. Here, we evaluate whether a novel potent and long-lasting ($t_{½}$ ≥7 hours) acylated α-CGRP analogue (αAnalogue) could alleviate and reverse cardiovascular disease in 2 distinct murine models of hypertension and heart failure in vivo.

METHODS: The ability of the αAnalogue to act selectively via the CGRP pathway was shown in skin by using a CGRP receptor antagonist. The effect of the αAnalogue on angiotensin II–induced hypertension was investigated over 14 days. Blood pressure was measured by radiotelemetry. The ability of the αAnalogue to modulate heart failure was studied in an abdominal aortic constriction model of murine cardiac hypertrophy and heart failure over 5 weeks. Extensive ex vivo analysis was performed via RNA analysis, Western blot, and histology.

RESULTS: The angiotensin II–induced hypertension was attenuated by cotreatment with the αAnalogue (50 nmol·kg^{-1}·d^{-1}, SC, at a dose selected for lack of long-term hypotensive effects at baseline). The αAnalogue protected against vascular, renal, and cardiac dysfunction, characterized by reduced hypertrophy and biomarkers of fibrosis, remodeling, inflammation, and oxidative stress. In a separate study, the αAnalogue reversed angiotensin II–induced hypertension and associated vascular and cardiac damage. The αAnalogue was effective over 5 weeks in a murine model of cardiac hypertrophy and heart failure. It preserved heart function, assessed by echocardiography, while protecting against adverse cardiac remodeling and apoptosis. Moreover, treatment with the αAnalogue was well tolerated with neither signs of desensitization nor behavioral changes.

CONCLUSIONS: These findings, in 2 distinct models, provide the first evidence for the therapeutic potential of a stabilized αAnalogue, by mediating (1) antihypertensive effects, (2) attenuating cardiac remodeling, and (3) increasing angiogenesis and cell survival to protect against and limit damage associated with the progression of cardiovascular diseases. This indicates the therapeutic potential of the CGRP pathway and the possibility that this injectable CGRP analogue may be effective in cardiac disease.

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Clinical Perspective

What Is New?

- We have used a novel injectable stabilized α-calcitonin gene–related peptide (CGRP) agonist, the first to our knowledge, to analyze the effect on cardiovascular disease in 2 distinct murine models.
- We show that the CGRP agonist is well tolerated and selective in the vasculature.
- The CGRP agonist prevented the onset of and limited angiotensin II–induced hypertension.
- The CGRP agonist also protected against heart failure in the abdominal aortic constriction model, suggesting that this α-CGRP analogue is beneficial in cardiovascular disease.

What Are the Clinical Implications?

- These preclinical data show that activating the protective CGRP pathway, using selective stabilized agonists, constitutes a novel therapeutic application.
- The results are a stimulus to further develop agonists, including those that are orally active.
- The results highlight the potential development of this injectable stabilized CGRP agonist (α-CGRP analogue) for the treatment of cardiac dysfunction (eg, in advanced heart failure).

Calcitonin gene-related peptide (CGRP) is a member of the calcitonin family of peptides. CGRP is primarily localized to sensory nerves, although nonneuronal sources are reported.[1] The major CGRP receptor is formed by the coexpression of calcitonin receptor-like receptor (CLR) with receptor activity–modifying protein-1 (RAMP1).[1] These receptors are found throughout the cardiovascular system, specifically in the media, intima, and endothelial layer of blood vessels. Although CGRP is established as a potent vasodilator, there is little evidence that sufficient endogenous CGRP is released to influence physiological blood pressure regulation, although CGRP-containing nerves surround all cardiovascular tissues. Several CGRP receptor antagonists and antibodies developed as migraine therapies have minimal effect on blood pressure in healthy individuals.[1,2] Evidence that CGRP plays a role in cardiovascular protection arises from acute studies where CGRP has been administered in rodent models of hypertension,[3,4] using spontaneously hypertensive rats[5,6] and α-CGRP–specific knockout (KO) mice.[7] The beneficial effects of the native CGRP peptide have also been observed when administered for up to 24 hours to patients with congestive heart failure with no evidence of tolerance.[8,9]

Although systemic endogenous CGRP levels are raised in some conditions such as pregnancy, it has proven difficult to raise endogenous CGRP levels to provide cardiovascular benefit. There is little evidence that stimulation of the major sensory nerve-localized transient receptor potential (TRP) channels (TRPV1 or TRPA1) releases CGRP to play a primary protective endogenous role in hypertension.[10,11] This is despite knowledge that activation of TRP channels expressed on sensory nerves induces CGRP-dependent vasodilation in peripheral tissues such as skin.[12] Of note, TRPA1 activation using nitroxyl[13] mediates inotropic effects in the failing heart,[14] but the importance of CGRP is debated.[15] Some CGRP/calcitonin KO mouse strains possess a raised blood pressure at baseline, indicating a potential role of endogenous CGRP.[1,16] We have shown that α-CGRP–specific KO mice have normal baseline blood pressure but enhanced hypertension following angiotensin II (AngII) infusion for up to 28 days, in comparison with wild-type mice.[7] This was associated with aortic hypertrophy and endothelial dysfunction observed as loss of endothelial nitric oxide synthase and evidence of oxidative stress.[7]

This and related knowledge, together with the understanding that global human RAMP1 transgenic mice are protected from hypertension,[17] led us to hypothesize that a stabilized α-CGRP agonist with the ability to remain active over prolonged periods would elicit cardioprotective properties. This first α-CGRP analogue (αAnalogue) is acylated with an albumin-binding fatty acid moiety that allows reversible albumin binding (patent WO 2011/051312 A1).[18,19] It has similar pharmacological properties to the native CGRP peptide, but exhibits prolonged action and improved pharmacokinetic properties with a half-life of >7 hours that benefited a model of type 2 diabetes mellitus.[18,19] The current study demonstrates that the stabilized αAnalogue[18] protects against the development of AngII-induced hypertension and abdominal aortic constriction (AAC)–induced cardiac hypertrophy and heart failure in mice for several weeks. We have determined mechanisms by which this αAnalogue can reverse vascular, renal, and cardiac damage. To our knowledge, this is the first detailed study using an α-CGRP agonist that has improved stability over the native peptide and illustrates the potential of the CGRP pathway as a therapeutic target and injectable stabilized CGRP agonists as therapeutic agents.

METHODS

A detailed Methods is provided in the online-only Data Supplement.

Animals

Experiments complied with ARRIVE (Animal Research: Reporting In vivo Experiments) guidelines, in accordance with the UK Home Office Animals (Scientific Procedures) Act, 1986 and approved by the local Animal Care and Ethics Committee.

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Male mice, CD1 or C57BL/6J (12–18 weeks of age; Charles River) were used.

AngII Murine Hypertension Model

Mice were infused with AngII (1.1 mg·kg^{-1}·d^{-1}) or saline (control) continuously for 14 days via osmotic mini pumps, as previously.[7] Mice were treated daily with αAnalogue (50 nmol·kg^{-1}·d^{-1}, SC) or vehicle (0.219 mol/L mannitol, 5% hydroxypropyl-β-cyclodextrin, 1.6% ammonium acetate at pH6.5) for 14 days or at 7 days onward for therapeutic dosing.

Cardiac Hypertrophy Murine Model

Mice were surgically subjected to pressure overload–induced cardiac hypertrophy and heart failure[20] for 5 weeks and treated daily with αAnalogue (50 nmol·kg^{-1}·d^{-1}, SC) or vehicle.

Measurement of Cutaneous Blood Flow

Blood flow was assessed in the ear, leg, or paw using the Full-field Laser Perfusion Imager (Moor Instruments) on anesthetized mice.[12] To investigate the local effects of αAnalogue, mice were pretreated with the CGRP antagonist BIBN4096 (0.3 mg/kg, IV) or control (neutralized saline) followed by αAnalogue injection (100 pmol daily, ipsilateral ear) or vehicle (contralateral ear). In separate experiments, blood flow in the periphery was measured following systemic treatment of the αAnalogue.

Measurement of Blood Pressure

Blood pressure, heart rate, and activity were measured using a radiotelemetry (PA-C10, DSI), as previously described[10–12] in AngII-infused C57BL/6J mice. For the characterization of the systemic dose of αAnalogue, blood pressure was measured by tail-cuff plethysmography (CODA 8, Kent Scientific) in conscious mice.[7,10] Following AAC-induced cardiac hypertrophy, blood pressure was measured via carotid artery in anesthetized mice.

Echocardiography

In vivo cardiac function was assessed using a Vevo 2100 Imaging System with a 40-MHz linear probe (Visualsonics).[20] Data analysis was performed with Vevo 2100 software v.1.2.1 (Visualsonics).

Light Aversion Assay

Light aversion (10 minutes, 1000 lux) was determined at baseline and 2 hours following injection of αAnalogue, vehicle, or

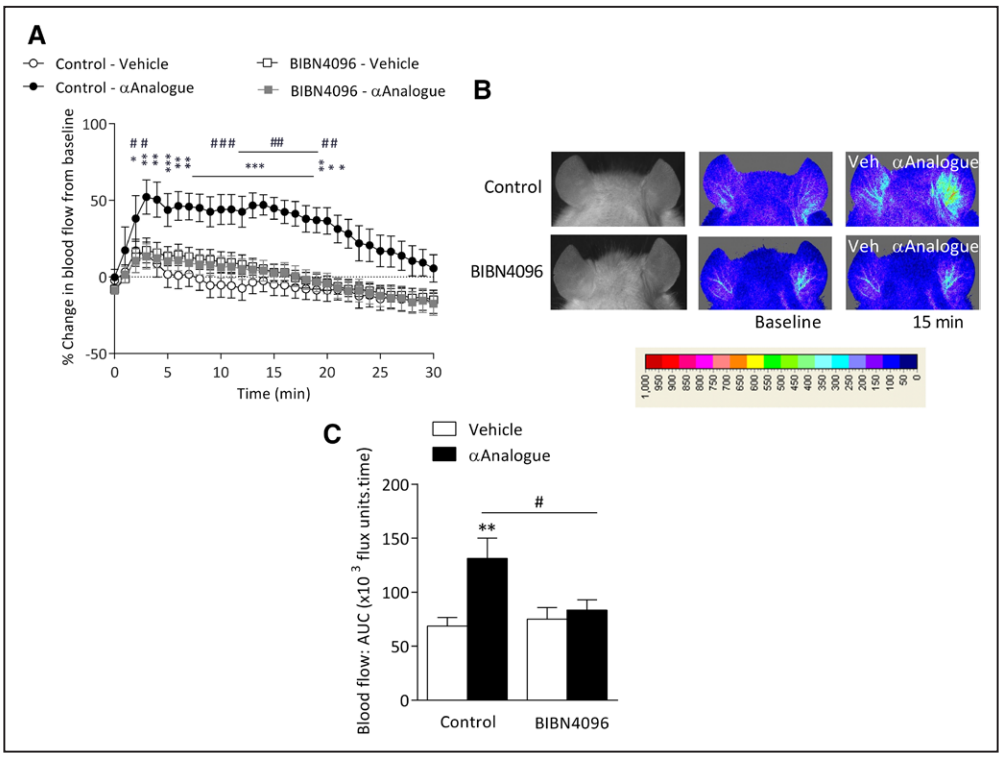

Figure 1. α-CGRP analogue (αAnalogue) increases vascular blood flow via CGRP receptors.
Blood flow monitored using Full-field Laser Perfusion Imager in the ear vasculature of mice pretreated with control (saline) or CGRP receptor antagonist BIBN4096 (0.3 mg/kg, IV) at baseline and following intradermal injection of αAnalogue (100 pmol, daily) or vehicle (Veh) (15 μL, n=6). **A**, Blood flow responses expressed as % change from baseline. **B**, Representative Full-field Laser Perfusion Imager pictures alongside gray/black photo showing blood flow at baseline and 15 minutes after treatment. **C,** Blood flow assessed by area under the curve (AUC) for 30 minutes following vehicle or αAnalogue administration (n=6). Data showed as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 versus vehicle-treated; #P<0.05, ##P<0.01, ###P<0.001 for αAnalogue treated (**A**, repeated-measures 2-way ANOVA + Bonferroni post hoc test; **C**, 2-way ANOVA + Bonferroni post hoc test). α-CGRP indicates α-calcitonin gene–related peptide.

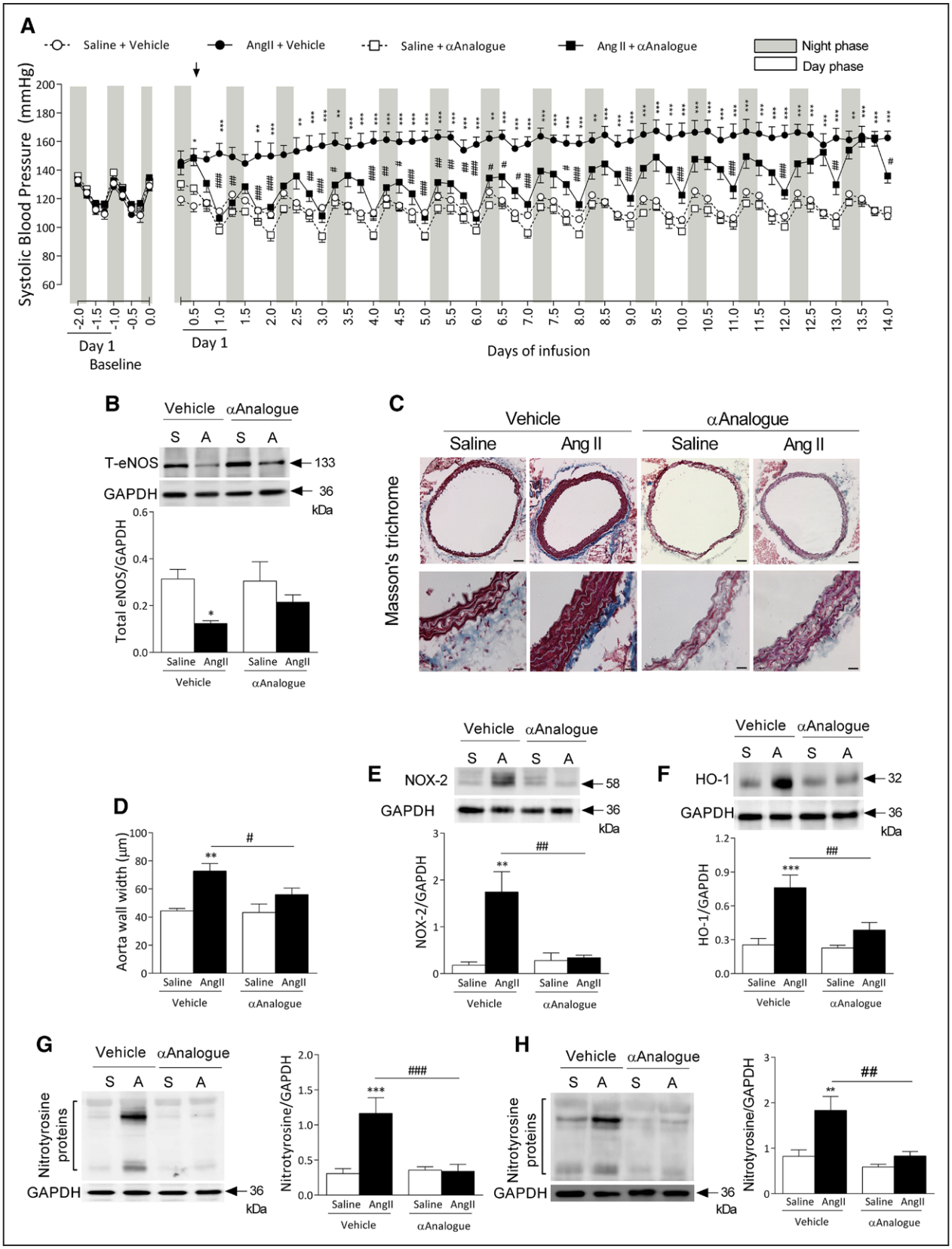

Figure 2. Daily systemic treatment with α-CGRP analogue (αAnalogue) protects against angiotensin II (AngII)–induced hypertension and vascular damage.

Mice were infused with AngII (**A**, 1.1 mg·kg^{-1}·d^{-1}) or control (S, saline) for 14 days and treated daily with vehicle (V) or αAnalogue (50 nmol/kg, SC). **A**, Systolic blood pressure was measured by radiotelemetry. Results expressed as 6-hour average. (*Continued*)

positive control glyceryl trinitrate (320 nmol/kg, IV). See the online-only Data Supplement.

Glucose Tolerance Test

Mice were fasted for 6 hours and treated with glucose (1 g/kg, IP). Blood glucose level was determined at baseline and stated time points using a One Touch Vita glucose meter (Lifescan).

RNA Preparation and Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted using the Qiagen RNeasy Microarray Mini Kit (Qiagen), followed by reverse transcription into cDNA (Applied Biosystems, Life technologies Ltd). Quantitative polymerase chain reaction was performed with a SYBR-green–based polymerase chain reaction mix (Sensi-Mix, SYBR-green No ROX; Bioline). Primer details are listed in online-only Data Supplement Table I.

Western Blotting

Western blot analysis was performed in aorta, mesentery, heart, and kidney as previously described.[7] Antibody details are listed in the online-only Data Supplement.

Quantification of Noradrenaline and Cytokines Using Enzyme-Linked Immunosorbent Assay

After 14 days of AngII or saline infusion, plasma and kidney samples were collected for determination of noradrenaline and inflammatory cytokines (interleukin-6, tumor necrosis factor-α) by using standard enzyme-linked immunosorbent assay as described in the online-only Data Supplement.

Histology

Aorta, heart, and kidney tissues were fixed in 4% paraformaldehyde, as previously described.[7] Staining protocol and antibody details are listed in the online-only Data Supplement.

Statistical Analysis

Results are expressed as mean±SEM. Statistical analysis was performed using an unpaired 2-tailed Student *t* test, 1-way or repeated-measures 2-way ANOVA followed by Bonferroni post hoc test. $P<0.05$ was considered to represent a significant difference.

RESULTS

Effects of Local Administration of the αAnalogue on Vascular Blood Flow

Initial studies determined the ability of the αAnalogue to increase blood flow via the CGRP receptor (CLR/RAMP1) pathway. Intradermal administration of the αAnalogue increased blood flow in a dose-dependent manner in the skin of anesthetized naive mice as determined by laser perfusion imaging (Figure 1A and 1B, online-only Data Supplement Figure I). This effect was abolished by the selective nonpeptide CGRP receptor antagonist BIBN4096 (Figure 1A through 1C).

Systemic Treatment With the αAnalogue Protects Against Hypertension

Systemic injection of the αAnalogue (10–100 nmol/kg, SC) induced a dose-dependent decrease in blood pressure at 1 to 6 hours, with significance observed at 100 nmol/kg in comparison with vehicle treatment, assessed by tail-cuff in naive mice (online-only Data Supplement Figure II). A 50 nmol/kg dose was chosen for further studies, because the hypotensive response had recovered by 24 hours in all mice. It is important to note that the blood pressure effects of the αAnalogue (50 nmol/kg, SC) were not significantly different from vehicle-treated mice (Figure 2A, online-only Data Supplement Figure III). All mice demonstrated normal diurnal variations in cardiovascular hemodynamics at baseline, followed by a hypertensive phenotype post–AngII infusion (Figure 2, online-only Data Supplement Figure III). Daily systemic treatment with the αAnalogue throughout the 14 days markedly blunted AngII-induced hypertension (Figure 2A, online-only Data Supplement Figure III). No significant change in heart rate or activity was observed among treatment groups (online-only Data Supplement Figure III).

In the AngII-infused mice, treatment with the αAnalogue leads to a reproducible reduction in blood pressure, with a similar reduction observed at day 1 to day 14 of treatment (online-only Data Supplement Figure IV), although there was a reduced hypotensive response to the αAnalogue on the last day. However, the protective activity was clearly maintained, with marked reduction in vascular remodeling and oxidative stress

Figure 2 Continued. Mice experience a 12/12 hour light/dark cycle, with the dark cycle shown in the gray striped area. Arrow represents the start of daily treatment. **B**, Protein expression of total eNOS in aorta (n=4–5). **C**, Representative images of Masson trichrome-stained aortic sections. **D**, Quantification of smooth muscle wall width (n=4–5; scale bars, 100 μm). Protein expression of NADPH oxidase-2 (NOX-2) (**E**), heme oxygenase-1 (HO-1) (**F**), nitrotyrosine in aorta (n=4–6) (**G**). **H**, Protein expression of nitrotyrosine in mesenteric vessels (n=6–7). Results shown as mean±SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus vehicle-treated saline-infused; #$P<0.05$, ##$P<0.01$, ###$P<0.001$ for αAnalogue-treated AngII-infused versus vehicle-treated AngII-infused (**A**, repeated-measures 2-way ANOVA + Bonferroni post hoc test; **B** through **H**, 2-way ANOVA + Bonferroni post hoc test). α-CGRP indicates α-calcitonin gene–related peptide; and eNOS, endothelial nitric oxide synthase.

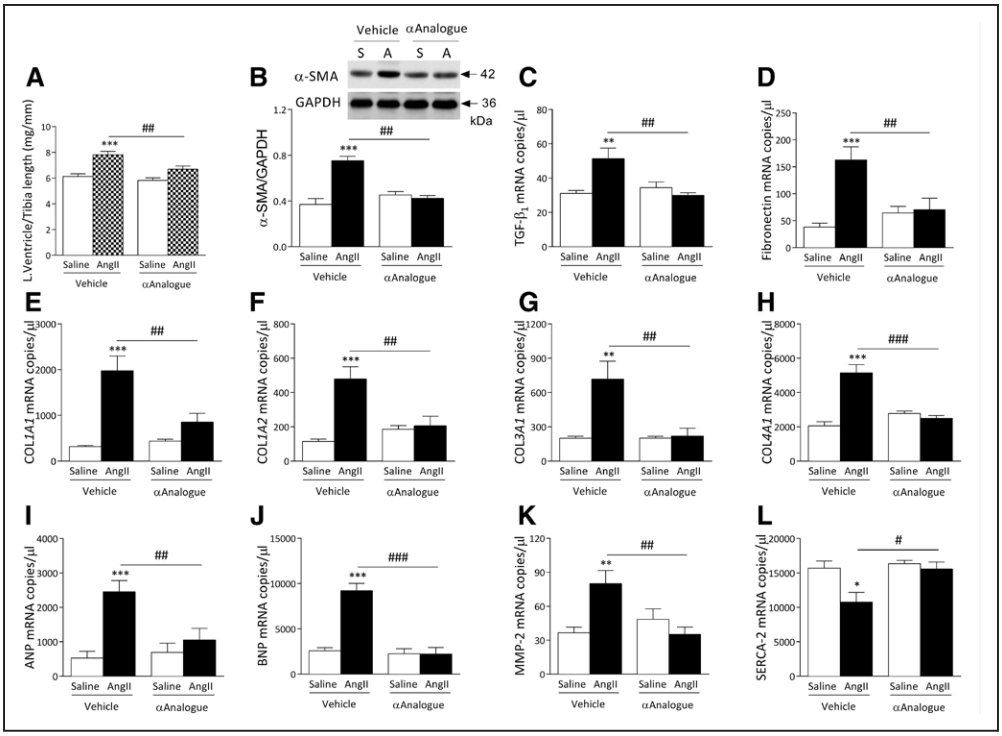

Figure 3. α-CGRP analogue (αAnalogue) protects against angiotensin II (AngII)–induced cardiac hypertrophy and fibrosis.

Mice were treated as in Figure 2 (AngII, A; Saline, S). **A**, Left ventricle weight normalized to tibia length ratio (mg/mm). **B,** Protein expression of α-smooth muscle actin (α-SMA) in heart (n=5). mRNA expression measured by real-time quantitative polymerase chain reaction for transforming growth factor-β1 (TGF-β_1) (**C**), fibronectin (**D**), collagen type 1 α1 (COL*1A1*) (**E**), collagen type 1 α2 (COL*1A2*) (**F**), collagen type 3 α1 (COL*3A1*) (**G**), collagen type 4 α1 (COL*4A1*) (**H**), atrial natriuretic peptide (ANP) (**I**), brain natriuretic peptide (BNP) (**J**), matrix metalloproteinase-2 (MMP-2) (**K**), and sarcoplasmic reticulum Ca^{2+} ATPase-2 (SERCA-2) (**L**) in heart (n=5–11). Results expressed as copy numbers per microliter normalized to hypoxanthine-guanine phosphoribosyltransferase, B_2M and β-actin, and showed as mean±SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 versus vehicle-treated saline-infused; #*P*<0.05, ##*P*<0.01, ###*P*<0.001 versus vehicle-treated AngII-infused (2-way ANOVA + Bonferroni post hoc test). α-CGRP indicates α-calcitonin gene–related peptide.

(Figure 2). AngII infusion increased water consumption from day 3, consistent with previous findings,[21] and this was reduced by the αAnalogue treatment throughout the time course (online-only Data Supplement Figure VA and VB). Typically, AngII reduced body weight, which was absent in αAnalogue-treated mice (online-only Data Supplement Figure VC). Food intake was not affected in any treatment groups (online-only Data Supplement Figure VD).

Systemic Treatment With the αAnalogue Does Not Affect Normal Behavioral Responses or Glucose Homeostasis

One of the limiting factors in the potential use of CGRP agonists therapeutically is that they may cause indications relevant to migraine, flushing, or metabolic changes.[1] We therefore examined its effects on activity and core body temperature by radiotelemetry, behavioral responses using a light aversion assay, and signs of flushing by assessing peripheral blood flow. Treatment with the αAnalogue (50 nmol/kg, SC) had no effect on activity (online-only Data Supplement Figure IIID) or light avoidance in comparison with baseline (online-only Data Supplement Figure VI), unlike glyceryl trinitrate, an established inducer of migraine symptoms. Neither acute nor chronic systemic treatment of the αAnalogue had a significant effect on skin blood flow (online-only Data Supplement Figure VII). We found no significant change in core body temperature (online-only Data Supplement Figure VIII) or in glucose homeostasis (online-only Data Supplement Figure IX) with the αAnalogue treatment in comparison with vehicle.

αAnalogue Protects Against AngII-Induced Vascular Changes in the Aorta and Mesentery

AngII infusion caused endothelial dysfunction, revealed by a significant decrease in endothelial nitric oxide synthase expression and vascular hypertrophy,

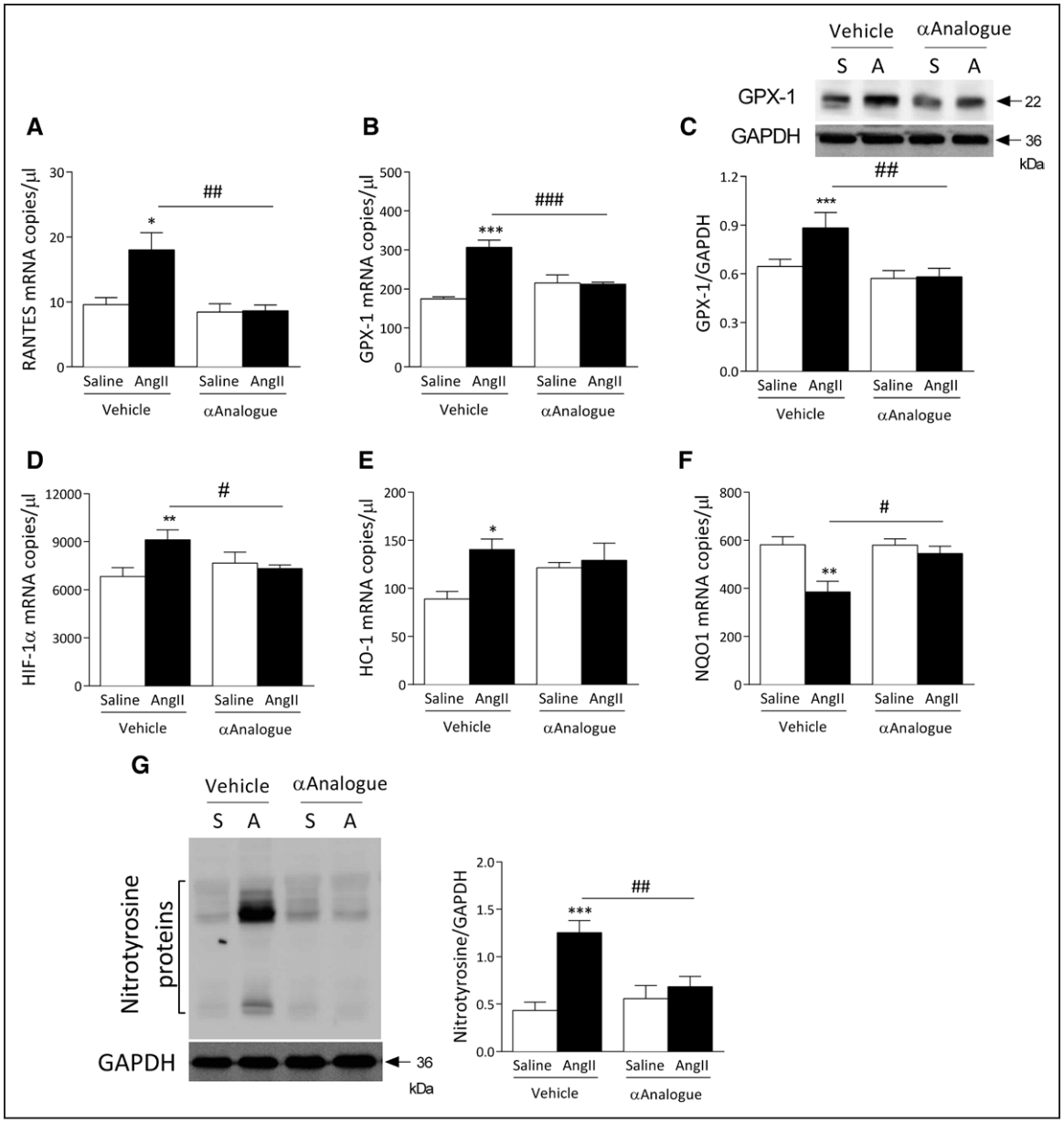

Figure 4. Daily systemic treatment with α-CGRP analogue (αAnalogue) protects against angiotensin II (AngII)–induced cardiac inflammation and oxidative stress.

Mice were treated as in Figure 2 (AngII, A; Saline, S). mRNA expression measured by real-time quantitative polymerase chain reaction (n=5–11) for RANTES (**A**) and glutathione peroxidase-1 (GPX-1) (n=6–11) (**B**). **C**, Protein expression of GPX-1 in heart (n=5). mRNA expression for hypoxia-inducible factor-1 (HIF-1α) (**D**), heme oxygenase-1 (HO-1) (**E**), and NADPH dehydrogenase quinone-1 (NQO1) (n=5–11) (**F**). Results expressed as copy numbers per microliter normalized to hypoxanthine-guanine phosphoribosyltransferase, B_2M and β-actin, and showed as mean±SEM. **G**, Protein expression of nitrotyrosine (n=5) in heart (n=6–7). **P*<0.05, ***P*<0.01, ****P*<0.001 versus vehicle-treated saline-infused; #*P*<0.05, ##*P*<0.01, ###*P*<0.001 versus vehicle-treated AngII-infused (2-way ANOVA + Bonferroni post hoc test). α-CGRP indicates α-calcitonin gene–related peptide.

with increased aortic wall thickness. These were not observed in mice treated with the αAnalogue (Figure 2B through 2D). AngII upregulated NADPH oxidase-2 (NOX-2) expression, accompanied by increased vascular aortic oxidative stress, as reflected by an increase in the stress-response protein heme oxygenase-1 (HO-1) and nitration of protein tyrosine residues, an indication of peroxynitrite formation in vehicle-treated AngII-infused mice. These responses were attenuated by the αAnalogue (Figure 2E through 2G). Although we observed no changes in endothelial nitric oxide synthase expression following AngII infusion in mesenteric vessels (online-only Data Supplement Figure X), there was a marked increase in nitrosative stress, which was blocked by the αAnalogue (Figure 2H).

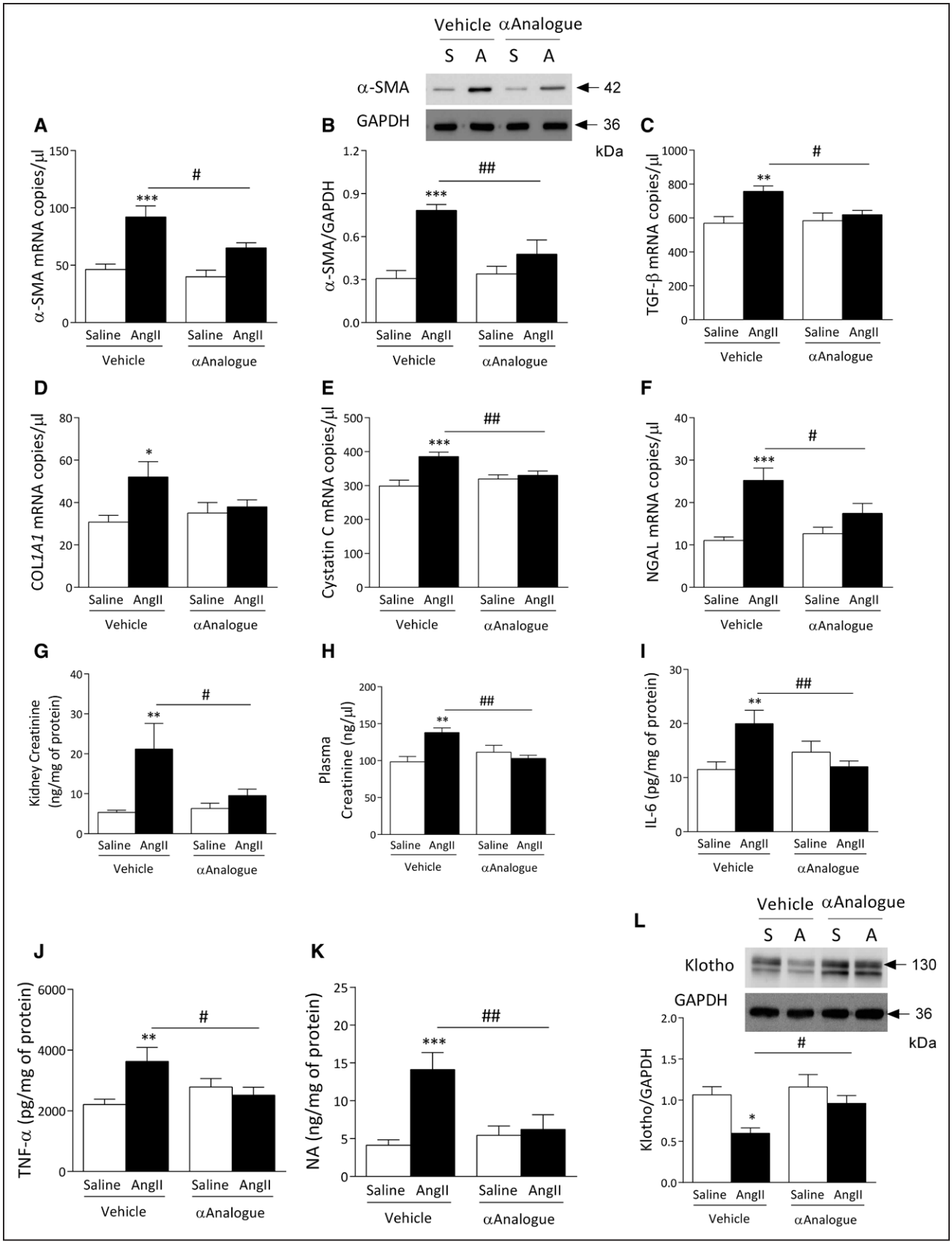

Figure 5. Daily systemic treatment with α-CGRP analogue (αAnalogue) protects against angiotensin II (AngII)–induced renal fibrosis, dysfunction, and inflammation.

Mice were treated as in Figure 2 (AngII, A; Saline, S). mRNA (**A**) and protein expression of α-SMA (**B**) in kidney. mRNA (*Continued*)

αAnalogue Treatment Protects Against AngII-Induced Cardiac Remodeling, Fibrosis, and Oxidative Stress

AngII-induced cardiac hypertrophy was reduced by the αAnalogue (Figure 3A, online-only Data Supplement Table II). Hearts of vehicle-treated but not αAnalogue-treated AngII-infused mice showed increased protein expression of α-smooth muscle actin and mRNA expression of several cardiac stress markers, including transforming growth factor-β_1, a major driver of cardiac fibrosis, extracellular matrix remodeling markers such as connective tissue growth factor, fibronectin, collagen type 1, 3, and 4, and natriuretic peptides such as atrial natriuretic peptide and brain natriuretic peptide (Figure 3B through 3J, online-only Data Supplement Table III). Transforming growth factor-β_1 induces matrix metalloproteinase-2 in fibroblasts contributing to cardiac remodeling.[22] Daily treatment with the αAnalogue protected against AngII-induced changes in matrix metalloproteinase-2 and the corresponding tissue inhibitor of metalloproteinase-2 mRNA expression in the hearts (Figure 3K, online-only Data Supplement Table III). The cardiac muscle–specific sarcoplasmic reticulum Ca^{2+} ATPase-2 is important in Ca^{2+} handling, and its expression decreases under pathological conditions, leading to contractile dysfunction.[23] The αAnalogue protected against AngII-induced decrease in sarcoplasmic reticulum Ca^{2+} ATPase-2 mRNA expression (Figure 3L).

The αAnalogue protected against AngII-induced inflammation, as shown by reduced mRNA expression of the nuclear factor kappa B cells and the chemokine RANTES (Figure 4A, online-only Data Supplement Table III). Treatment with the αAnalogue caused no change in the apoptosis regulator B-cell lymphoma-2 (Bcl-2) but resulted in a significant reduction in apoptotic markers such as p53 in AngII-infused hearts (online-only Data Supplement Table III). We next determined the effects of the αAnalogue on oxidative stress. AngII-induced increase in the antioxidant enzyme glutathione peroxidase-1 expression was absent in αAnalogue-treated groups (Figure 4B and 4C). An increase in hypoxia-inducible factor 1α was attenuated by αAnalogue treatment (Figure 4D). AngII-induced changes in the mRNA expression of HO-1 and the phase II detoxifying enzymes NADPH dehydrogenase quinone-1 were absent by daily treatment with the αAnalogue (Figure 4E and 4F). Cardiac nitrosative stress was absent by the αAnalogue treatment in AngII-infused mice (Figure 4G). Collectively, these results show a marked protective effect on the proinflammatory and oxidative pathways associated with AngII-induced cardiovascular disease.

αAnalogue Protects Against AngII-Induced Renal Fibrosis and Injury

AngII infusion caused renal fibrosis, indicated by upregulation of α-smooth muscle actin expression, increased transforming growth factor-β_1 and collagen mRNA expression, which were absent in mice cotreated with the αAnalogue (Figure 5A through 5D). The kidneys of AngII-infused mice exhibited renal damage, with increased mRNA expression of cystatin C and neutrophil gelatinase-associated lipocalin, plasma and renal creatinine levels, and mesangial matrix expansion in the glomeruli, which were all absent in mice treated with the αAnalogue (Figure 5E through 5H, online-only Data Supplement Figure XI). AngII infusion resulted in renal inflammation, highlighted by increased interleukin-6 and tumor necrosis factor-α concentrations, which were abolished by the αAnalogue (Figure 5I and 5J). There was a significant increase in localized sympathetic nerve activity, shown by increased noradrenaline content in AngII-infused kidney but not plasma, which was blocked by αAnalogue treatment (Figure 5K, online-only Data Supplemental Figure XII). The decreased noradrenaline levels in the kidney provides evidence that CGRP was able to attenuate the sympathetic activity. Downregulation of renal klotho expression can aggravate AngII-induced renal damage.[24] Here, the αAnalogue protects against this downregulation (Figure 5L).

αAnalogue Protects Against AngII-Induced Upregulation of CGRP Receptor RAMP1

RAMP1 determines the CGRP receptor phenotype when associated with CLR, and CGRP receptors are upregulated in cardiovascular disease models.[1] We observed a significant increase in CGRP receptor RAMP1 protein expression in the aorta, mesentery, and heart of vehicle-treated AngII-infused mice but not the αAnalogue

Figure 5 Continued. expression of TGF-β_1 (**C**), collagen type 1a1 (COL*1A1*) (**D**), cystatin C (**E**), and neutrophil gelatinase-associated lipocalin (NGAL) (**F**) in kidney. Creatinine levels in kidney (**G**) and plasma (**H**) (n=6–8). IL-6 (**I**), TNF-α (**J**), and noradrenaline (NA) (**K**) levels in kidney (n=6–9). **L,** Protein expression of klotho in kidney. mRNA expression measured by real-time quantitative polymerase chain reaction (n=6–8), expressed as copy numbers per microliter normalized to hypoxanthine-guanine phosphoribosyltransferase, B_2M, and β-actin. Results shown as mean±SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 versus vehicle-treated saline-infused; #*P*<0.05, ##*P*<0.01 versus vehicle-treated AngII-infused (2-way ANOVA + Bonferroni post hoc test). α-CGRP indicates α-calcitonin gene–related peptide; IL-6, interleukin 6; α-SMA; α-smooth muscle actin; TGF-β1; transforming growth factor-β1; and TNF-α, tumor necrosis factor-α.

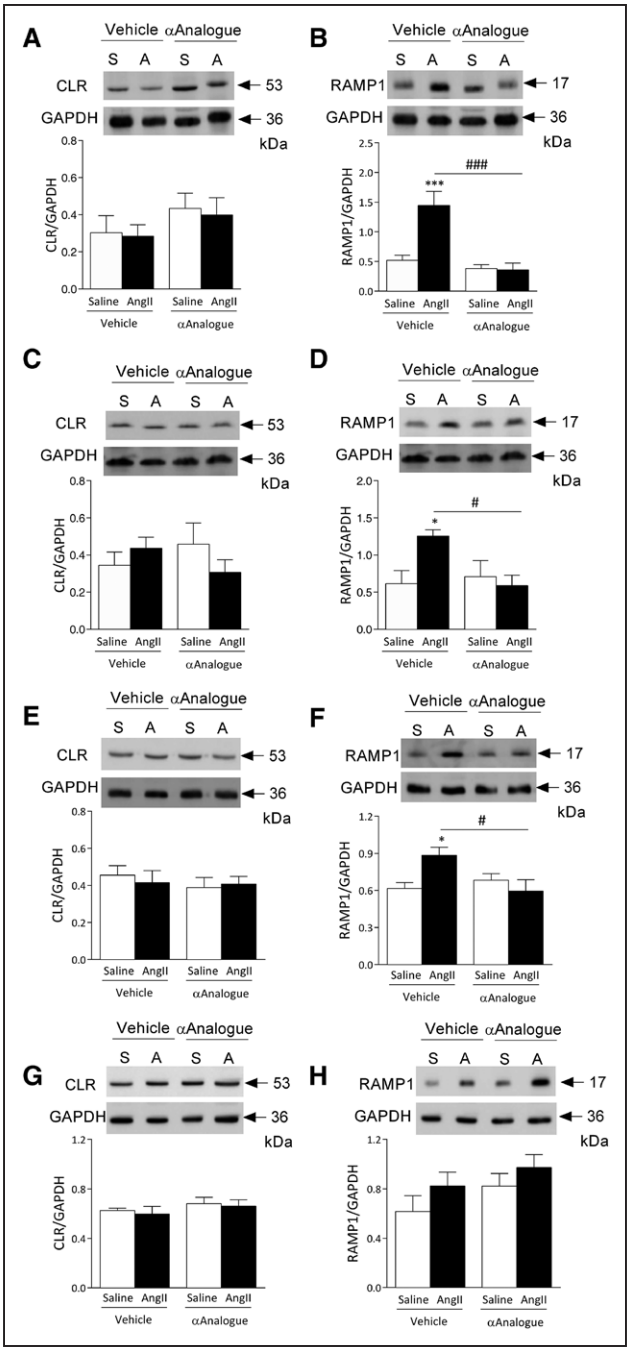

Figure 6. Effects of daily systemic treatment with α-CGRP analogue (αAnalogue) on CGRP receptor expression in angiotensin II (AngII)–induced hypertension. Mice were treated as in Figure 2 (AngII, A; saline, S, V, vehicle; α-CGRP analogue; C). Protein expression of calcitonin receptor-like receptor (CLR) (**A**) and receptor-associated membrane protein-1 (RAMP1) (**B**) in aorta (n=6). Protein expression of CLR (**C**) and RAMP1 (**D**) in mesenteric vessels (n=6). Protein expression of CLR (**E**) and RAMP1 (**F**) in heart (n=6–7). Protein expression of CLR (**G**) and RAMP1 (**H**) in kidney (n=5–6). Results showed as mean±SEM.*P<0.05, ***P<0.001 versus vehicle-treated saline-infused; #P<0.05, ###P<0.001 versus vehicle-treated AngII-infused (2-way ANOVA + Bonferroni post hoc test). α-CGRP indicates α-calcitonin gene–related peptide.

(Figure 6). There was no significant change in CLR expression observed in different tissues, regardless of treatment (Figure 6). We found no change in endothelin-1 expression in heart or aorta treated with vehicle or the αAnalogue (online-only Data Supplement Figure XIII).

αAnalogue Limits AngII-Induced Hypertension and Associated Cardiac Remodeling

To determine whether the αAnalogue influences established hypertension, we assessed its effects by starting treatment at day 7 of AngII infusion. Treatment with the αAnalogue attenuated AngII-induced hypertension (Figure 7A, online-only Data Supplement Figure XIV). We observed a significant decrease in Akt and osteopontin, remodeling, and fibrotic markers (transforming growth factor-β_1, connective tissue growth factor, α-smooth muscle actin, collagen type 1 and 3), NOX-2, and markers of oxidative stress (HO-1, NADPH dehydrogenase quinone-1) in aorta (online-only Data Supplement Table IV). Accordingly, a substantial decrease in cardiac hypertrophy was found, with a significant reduction in left ventricular weight:tibia length ratio (Figure 7B, online-only Data Supplement Table V) and in cardiac fibrosis, remodeling, and inflammation, shown by a significant reduction in collagen type 3, atrial natriuretic peptide, and NOX-2 mRNA expression (online-only Data Supplement Table VI). It is notable that the αAnalogue reduced AngII-mediated increase in cardiomyocyte size and collagen deposition, revealed by wheat germ agglutinin and Picrosirius Red staining, respectively (Figure 7C and 7D). Cardiac nitrosative stress was reversed by the αAnalogue in hypertensive mice (Figure 7E).

αAnalogue Protects Against AAC-Induced Cardiac Hypertrophy and Heart Failure

To investigate the cardioprotective effect of CGRP in heart failure, mice were subjected to sham or AAC-induced cardiac hypertrophy and heart failure for 5 weeks. Quantification of in vivo cardiac function revealed that ejection fraction was better preserved in αAnalogue-treated AAC mice, with a reduction in septum wall thickness as shown by echocardiography (Figure 8A and 8B, online-only Data Supplement Figure XV, and online-only Data Supplement Table VII). Chronic treatment with the αAnalogue for 5 weeks was well tolerated, with no significant change in body weight, food and water intake, light avoidance, or blood pressure (online-only Data Supplement Figures XVI and XVII). αAnalogue-treated mice consistently developed less cardiac hypertrophy and fibrosis after AAC than vehicle-treated groups (Figure 8C, 8E through 8H, online-

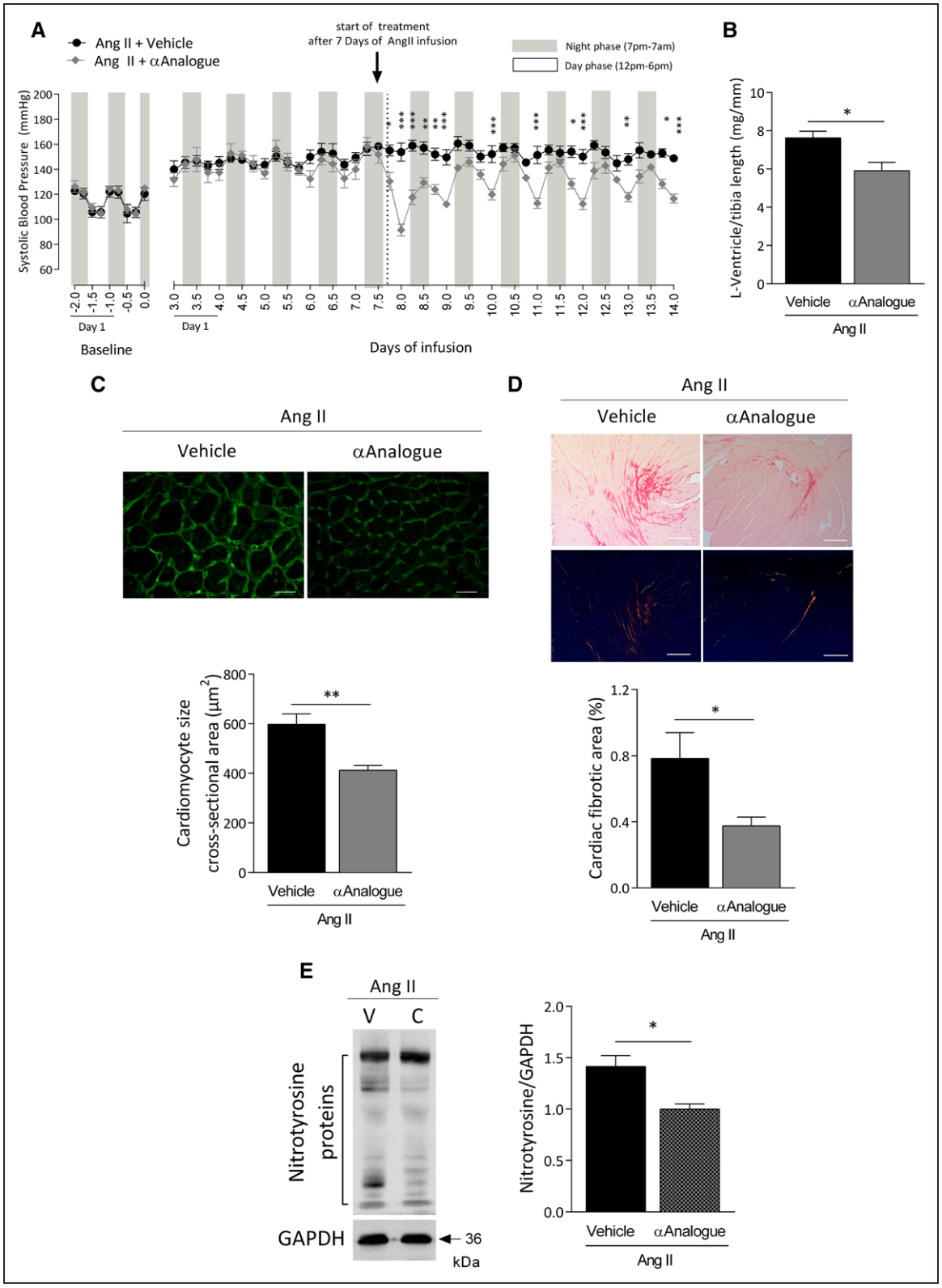

Figure 7. α-CGRP analogue (αAnalogue) limits angiotensin II (AngII)–induced hypertension.
Mice were infused with AngII (1.1 mg·kg^{-1}·d^{-1} for 14 days) and treated daily with vehicle or αAnalogue (50 nmol/kg, SC) at day 7 to 14 of infusion (n=4). **A**, Systolic blood pressure was measured by radiotelemetry. Results expressed as 6-hour average. Mice experience a 12/12 hour light/dark cycle, with the dark cycle shown in the gray striped area. Arrow represents the start of daily treatment. **B**, Left ventricle weight normalized to tibia length ratio (mg/mm). Representative heart sections (**Top**) and analysis (**Bottom**) showing cardiac hypertrophy by cardiomyocyte borders outlined using wheat germ agglutinin (scale (*Continued*)

only Data Supplement Table VIII, and online-only Data Supplement Figure XVIII). Insufficient angiogenesis is known as a driver of heart failure,[20] and left ventricular heart sections subjected to AAC showed reduced capillary density that was reversed by αAnalogue treatment (Figure 8I and 8J). Quantitative immunoblotting showed that phosphorylated p38 mitogen-activated protein kinase levels were significantly increased in αAnalogue-treated AAC hearts (online-only Data Supplement Figure XIX). Mice subjected to AAC showed an increase in cardiac inflammation, oxidative, nitrosative stress, and apoptosis in vehicle-treated but not αAnalogue-treated groups (Figure 8K through 8N, online-only Data Supplement Figure XVIII).

DISCUSSION

CGRP is one of the most potent microvascular dilators known, and its protective properties are well established in vitro. However, it has been difficult to harness this information in drug discovery projects involving the cardiovascular system to date, primarily because of the instability of the peptide. This study presents novel mechanistic evidence that chronic systemic treatment with an injected stabilized α-CGRP agonist is effective in the onset and ongoing AngII-induced hypertension and AAC-induced cardiac hypertrophy and heart failure in vivo. The αAnalogue, which possesses a considerably longer half-life than the native CGRP peptide (>7 hours in comparison with <30 minutes) is well tolerated. Specifically, the agonist did not lower systemic blood pressure in naive mouse, but exerted beneficial protective effects at the vascular, renal, and cardiac levels by alleviating fibrosis, remodeling, inflammation, oxidative stress, apoptosis, and preserving overall function in 2 distinct cardiovascular disease models. Our results are consistent with the concept that the CGRP receptor can be an influential regulatory component in cardiovascular disease and that CGRP agonists have potential therapeutic benefits.

The αAnalogue acted as a selective CGRP agonist via the CGRP receptor to mediate vasodilatation. Because the αAnalogue is not orally active, a systemic injectable dose that elicited an initial decrease in blood pressure with full recovery by 24 hours was chosen. This allowed baseline blood pressure to be maintained over the 2- to 5-week protocols in the control/sham mice. Although systemic CGRP infusion previously led to flushing in humans,[25] we found no undesirable side effects at the selected dose.

Although α-CGRP–specific KO mice have normal basal cardiovascular hemodynamics, they exhibit an enhanced hypertensive phenotype in AngII-induced hypertension.[7] Indeed, acute injection of the native CGRP peptide was beneficial in hypertensive rats[26] but effects were short lasting (<10 minutes) because of its short half-life.[27] In contrast, CGRP infusion for 6 days had beneficial effects in hypertensive rats.[3] To build on this and elucidate the mechanisms, we show that the αAnalogue protects against AngII-induced increase in blood pressure for 2 weeks. We tested for potential desensitization by the daily administration of the αAnalogue in naive mice and found a reproducible hypotensive effect, with no signs of downregulation of the CGRP pathway in the vasculature. However, there was a reduced effect on hypertension at the 14-day time point, leading us to investigate for other markers of disease. AngII-induced hypertension is associated with weight loss and increased water intake, in addition to hypertension. These were reversed by the αAnalogue throughout. Of interest, blocking the CGRP pathway with antagonists/antibodies against CGRP and its receptor (currently in clinical trials for prevention of migraine[2]) may interfere with this protective pathway and thereby increase cardiovascular risk. However, in the limited clinical studies performed to date, no evidence has been found. It is possible that endogenous levels of CGRP do not reach sufficient levels in humans to mediate protective effects. To our knowledge, this is the first in-depth study in cardiovascular models with a long-acting CGRP agonist where there has been a sustained benefit in protective cardiovascular effects.

AngII-induced vascular dysfunction and remodeling were reversed by the αAnalogue, in keeping with reports that CGRP inhibits smooth muscle cell proliferation by increasing cAMP or inhibiting the ERK1/2 signaling cascade.[28] CGRP has direct anti-inflammatory and antioxidant effects in endothelial progenitor cells.[29] Accordingly, the αAnalogue reduced the AngII-induced increase in the endogenous source of ROS[30] NOX-2, oxidative stress-associated proteins, and nitrosative stress in hypertensive aorta, which was consistent with less nitrosative stress in the hypertensive mesenteric vessels. There was no change in vascular endothelial dysfunction in resistance vessels, complementing our previous findings.[7]

CGRP receptors are upregulated in cardiovascular disease,[1] with evidence of a pressure-dependent regulation.[12] This may amplify responses to CGRP, especially in hypertension.[7,17] Accordingly, RAMP1 expression was increased in hypertensive resistance and conduit vessels. The αAnalogue reduced this effect such that RAMP1 returned to normal expression levels as the cardiovas-

Figure 7 Continued. bars, 20 μm) (**C**) and cardiac fibrosis by Picrosirius Red staining (scale bars, 200 μm) (**D**). **E**, Protein expression of nitrotyrosine in heart. Results showed as mean±SEM.*P<0.05, **P<0.01, ***P<0.001 versus vehicle-treated (**A**, repeated-measures 2-way ANOVA + Bonferroni post hoc test; **B** through **E**, 2-way ANOVA + Bonferroni post hoc test). α-CGRP indicates α-calcitonin gene–related peptide.

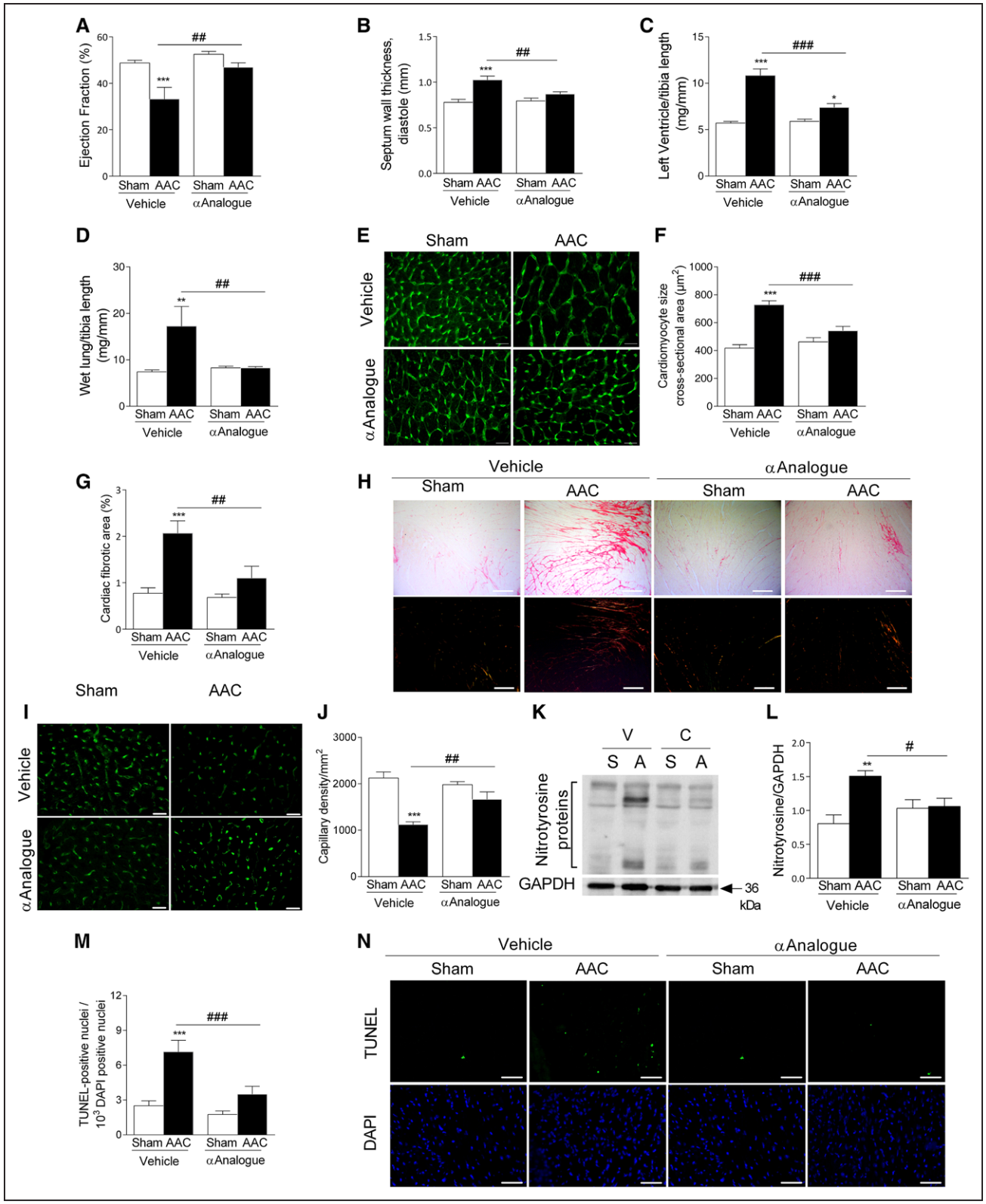

Figure 8. α-CGRP analogue (αAnalogue) preserves heart function post–AAC-induced cardiac hypertrophy and heart failure.

Mice were treated daily with vehicle and αAnalogue (50 nmol/kg, SC) postsurgery for 5 weeks (n=6–8). Ejection fraction (%) (**A**), septum wall thickness at diastole (mm) (**B**), left ventricle mass normalized to tibia length (mg/mm) (**C**), and wet lung mass (mg) normalized to tibia length **(D)**. Representative images (**E**) and quantification of cardiomyocyte cross-sectional area (**F**) in heart using wheat germ agglutinin staining (scale bars, 20 μm). Quantification (**G**) and representative images (*Continued*)

cular system benefited, irrespective of the continuing presence of AngII. This finding suggests that CGRP may act in an autoregulatory manner depending on CGRP peptide/agonist availability.

CGRP has positive chronotropic effects,[31] and α-CGRP KO mice exhibit exacerbated cardiac dysfunction in pressure overload–induced hypertrophy[32] and deoxycorticosterone acetate salt–induced hypertension.[33,34] Here, the αAnalogue had no effect on in vivo heart function, as observed by radiotelemetry or echocardiography in sham mice, but protected against cardiac hypertrophy in AngII-induced hypertension. The αAnalogue downregulated markers of fibrosis, remodeling, and hypertrophy, and protected against the reduction in the cardiac contractile dysfunction marker sarcoplasmic reticulum Ca^{2+} ATPase-2 in hypertensive hearts, as well. Our results correlate with previous in vitro findings[35–37] and provide in vivo evidence that the αAnalogue has influential antiremodeling effects in the stressed heart, while not directly modulating normal heart activity in control mice.

The αAnalogue reduces cardiac inflammation, as shown by reducing AngII-induced increase in the chemokine RANTES. AngII increases hypoxia-inducible factor 1α mRNA expression, indicating the involvement of fibrotic and oxidative stress pathways, and this was reduced by the αAnalogue. CGRP regulates oxidative stress by the PI3K/Akt and mitogen-activated protein kinase signaling pathways,[38] and deletion of the CGRP gene exacerbates cardiac oxidative stress in ischemia-reperfusion injury.[39] The αAnalogue protects against AngII-induced changes in the endogenous antioxidant defense responses and nitrosative stress. A link between CGRP and HO-1 is known.[40] Activation of HO-1 and NADPH dehydrogenase quinone-1 can counteract hypertension, potentially through generating the vasodilator carbon monoxide[41,42] and improved endothelial nitric oxide synthase coupling.[43] Our data build on earlier findings in cardiomyocytes, smooth muscle cells, and α-CGRP KO mice, implying that the αAnalogue acts directly via its receptors on the heart and reduces stress-activated kinases induced by oxidative stress,[44] adverse tissue remodeling to preserve cardiac function.

Hypertension is associated with renal fibrosis and inflammation that were reduced by the αAnalogue, complementing knowledge that CGRP promotes renal protection in hypertension independently of its vasodilator action.[33,45] The αAnalogue preserved renal function by reducing mesangial matrix expansion, plasma and kidney creatinine levels, and expression of cystatin C and neutrophil gelatinase-associated lipocalin, the early biomarker of acute kidney injury. AngII increases renal adrenergic receptors[46] and we found a significant increase in renal noradrenaline content, which was reversed by the αAnalogue. Hence, the αAnalogue may preserve renal function by reducing sympathetic activity. Klotho has nephroprotective effects, and AngII-induced downregulation of klotho aggravates renal damage in hypertension.[24] This downregulation was rescued by the αAnalogue, and our results further confirm the link between klotho and CGRP.[29] The upregulation of klotho associated with the use of αAnalogue may translate into enhanced renal protection in hypertension. The primary functional source of CGRP was reported to be independent of the kidney, because chemical denervation of renal sensory afferents removed tissue CGRP but without any beneficial effect in hypertensive mice.[46] However, our study highlights that mimicking sensory nerve efferent function by using an αCGRP analogue protects against end-organ damage in hypertension, especially renal fibrosis, a strong predictor of clinical progression of kidney disease.

Strikingly, the αAnalogue limits AngII-induced cardiovascular pathologies, ameliorating cardiac remodeling and fibrosis. This highlights the therapeutic potential of the αAnalogue when given in established hypertension. Although the αAnalogue was unable to reduce vascular endothelial dysfunction, it reduced markers of damage such as osteopontin, the downstream regulator of Akt activity,[47] remodeling, fibrosis, and oxidative stress.[7] The αAnalogue reduced both vascular and cardiac NOX-2 expression. It is possible that many of the beneficial effects of the αAnalogue in both early or late onset of hypertension are primarily related to a decrease in blood pressure and the resultant reduction in pressure-induced damage in the vasculature, kidney, and heart. Although it is presently difficult to separate the direct effects of the αAnalogue on pressure and cardiac function, it is evident that CGRP has antihypertensive effects and induces protection in a comprehensive manner.

To investigate the cardioprotective effects of the αAnalogue, we subjected mice to AAC and examined cardiac function. The adverse structural remodeling and hypertrophy at 5 weeks post-AAC, with significant reduction in ejection fraction and increasing heart mass with fibrosis, were markedly attenuated in αAnalogue-treated mice, under conditions where blood pressure was similar to αAnalogue and

Figure 8 Continued. of fibrosis in heart (**H**) using picrosirius red staining (scale bars, 200 μm). Representative images (**I**) and quantification of capillary density in heart (**J**) using isolectin-B_4 staining (scale bars, 20 μm). **K** and **L,** Protein expression of nitrotyrosine in heart. Quantification (**M**) and representative images of apoptosis (**N**) using TUNEL staining (scale bars, 50 μm). Results showed as mean±SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 versus vehicle-treated sham mice; #*P*<0.05, ##*P*<0.01, ###*P*<0.001 versus vehicle-treated AAC mice (2-way ANOVA + Bonferroni post hoc test). AAC indicates abdominal aorta constriction; α-CGRP, α-calcitonin gene–related peptide; and TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

vehicle-treated sham mice. This is consistent with findings where α-CGRP/calcitonin KO mice showed adverse cardiac dysfunction with increased mortality following transverse aortic constriction.[32] Although CGRP infusion for 24 hours previously improved cardiac performance in patients with chronic congestive heart failure,[8,9] this is the first demonstration that chronic treatment with a long-lasting CGRP agonist is beneficial and well tolerated. Myocyte apoptosis is well documented in heart failure and CGRP regulates cell survival signaling and antiapoptotic pathway via CLR/RAMP1 in cardiomyocytes.[38,48] Although the αAnalogue reduces nuclear factor kappa B cells and apoptotic marker p53 expression in our acute AngII model, here, the αAnalogue reduced apoptosis, as revealed in terminal deoxynucleotidyl transferase dUTP nick-end labeling staining with increased p38 mitogen-activated protein kinase phosphorylation in the hypertrophic heart. Similarly, the αAnalogue maintained its antioxidant effects. Development of cardiac hypertrophy is associated with increased cardiomyocyte size and reduced capillary density, which ultimately lead to hypoxia and cell death.[20] These changes were markedly reduced by the αAnalogue. This finding supports earlier studies where CGRP acted as a proangiogenic factor[32,49] and suggests that CGRP promotes normal cardiac microvessel development, perhaps via angiogenesis in hypertrophic hearts. Further gene expression studies on ventricle tissues demonstrate that markers of heart failure were abrogated by the αAnalogue.

To our knowledge, this is the first study demonstrating that a long-lasting and stable α-CGRP agonist has the potential to act as a novel therapeutic agent, targeting key mechanisms to benefit cardiovascular dysfunction, with limited adverse pathological changes and side effects. The results support the concept provided by in vitro studies that CGRP protects against adverse remodeling, inflammation, oxidative stress, apoptosis, and end-organ damage in cardiovascular disease, in addition to its vasodilator activity. We thus propose that the CGRP pathway is a therapeutic target for the clinical treatment of cardiovascular disease and, more specifically, that injectable CGRP agonists may benefit heart failure.

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DISCLOSURES

None.

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FOOTNOTES

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A Novel α-Calcitonin Gene-Related Peptide Analogue Protects Against End-Organ Damage in Experimental Hypertension, Cardiac Hypertrophy, and Heart Failure

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Animals

Male mice, CD1 (#022) or C57BL/6J (#027) (12-18 weeks of age), purchased from Charles River (Kent, UK) were used in all experiments. Blood flow experiments were performed on CD1 mice, whilst all other experiments with C57BL/6J mice. Mice were housed in a climatically controlled environment, on a 12-h light/dark cycle, with free access to water and standard food *ad libitum*. All experiments were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act, 1986 and were approved by the King's College London Animal Care and Ethics Committee. All experiments were conducted in a blinded manner. Animals were randomly assigned to control or treatment groups and the experimenter was blinded towards treatment at the time of experiment.

Cutaneous blood flow measurement by Full-field Laser Perfusion Imager

Cutaneous blood flow was assessed in the whole area of ear, leg or paw area using the Full-field Laser Perfusion Imager (FLPI, Moor Instruments) on anaesthetised mice in their ventral positioned placed on a heating mat, maintained at 36°C, as previously described.[1-3]

Local effects of α-CGRP analogue (αAnalogue, Figure 1, Supplemental Figure 1): CD1 mice were anaesthetised intraperitoneally (*i.p.*) with ketamine (75 mg/kg) and medetomidine (1 mg/kg).[1] Following recording of baseline blood flow in both ears to ensure the haemodynamic vascular responses have stabilised after anaesthesia, mice were injected intravenously (*i.v.*) with vehicle (neutralised saline) or CGRP receptor antagonist (BIBN4096, 0.3mg/kg)[1, 4, 5] and blood flow was resumed for another 5 min. The ipsilateral ear was then injected intradermally (*i.d.*) with αAnalogue[6] (100pmol in 15µl, Novo Nordisk), whilst the contralateral ear received vehicle (0.219M Mannitol, 5% HPCD, 1.6% ammonium acetate at pH6.5). Initial experiments investigated other doses of αAnalogue (1-100pmol in 15µl). Changes in blood flow after these treatments were followed for 30 min. Results are expressed as (1) a measure of maximum % increase in blood flow from baseline or (2) arbitrary flux units ($\times 10^3$ flux units) measured as area under the recorded flux (response curve) versus time for the entire recording period for 30 min following *i.d.* injection.

Systemic effects of αAnalogue (Supplemental Figure 7): C57BL/6J mice were anaesthetised with isoflurane (2%, Abbott Laboratories) in 2l/min O_2 and blood flow in the ear was measured for 5 min. Mice were allowed to recover and injected subcutaneously (*s.c.*) with vehicle (0.219M Mannitol, 5% HPCD, 1.6% ammonium acetate at pH 6.5) or αAnalogue (50nmol/kg, *s.c.*, Novo Nordisk). At 1h following treatment, mice were anaesthetised and blood flow was measured for a further 5 min.

In other experiments, blood flow was measured for 5 min in the paw, leg and ear of mice treated daily with vehicle or αAnalogue (50nmol/kg, *s.c.*, Novo Nordisk) for 14 days. Results are expressed as an average blood flow (flux units) for the 5 min for each area.

Measurement of blood pressure via tail-cuff plethysmography

Blood pressure was measured by tail-cuff plethysmography, using the CODA 8 non-invasive blood pressure acquisition technique system for mice (Kent Scientific), as previously described.[7, 8] All measurements were taken at a thermoneutral ambient room temperature (25-27°C). C57BL/6J mice were warmed on a heating pad for 10 min prior to and during blood pressure recordings. Mice were trained for at least 10 consecutive days prior to baseline blood pressure measurements, to reduce stress-induced changes caused by restraint.

Dose-response characterisation of αAnalogue: Measurements were taken at baseline and 1, 6, 24 and 30h following administration of vehicle (0.219M Mannitol, 5% HPCD, 1.6% ammonium acetate at pH 6.5) or αAnalogue (10-100nmol/kg, *s.c.*, Novo Nordisk).

Measurement of blood pressure via carotid artery cannulation

After 5 weeks of abdominal aortic construction (AAC)-cardiac hypertrophy and heart failure, mice were anaesthetised using isoflurane (2%, Abbott laboratories, UK) in 2L/min O_2, where systemic blood pressure was monitored invasively[9]. The core temperature of the mice was maintained at 37°C with a homeothermic blanket (Havard apparatus, Cambridge, UK). Briefly, the left carotid artery was isolated and fluid-filled (heparin; 100U/ml diluted in 0.9% saline) cannula with an outer diameter of 0.7mm and internal diameter of 0.28mm (Smiths Medical-Portex, Hythe, UK) was introduced into the carotid artery. After a 5-min stabilisation period, systemic pressures were measured using PowerLab data acquisition system and LabChart 8 Pro software (ADInstruments Ltd, Oxfordshire, UK). Data was represented as an average of 10 min continuous recording.

Measurement of blood pressure via radiotelemetry

Blood pressure, heart rate and activity were measured using a radiotelemetry device (PA-C10, DSI, NL), with the catheter placed in the left carotid artery and advanced towards the aortic arch, as previously described[1, 8, 10] in C57BL/6J mice. The catheter was secured using surgical braided silk (5.0, waxed, Pearsalls sutures) and the outer wound closed with absorbable sutures (5.0, Ethicon, Johnson and Johnson) in a discontinuous pattern. The transmitter was placed *s.c.* in the right flank and the transmitter pocket was irrigated with sterile saline (0.9% saline; sodium chloride, pyrogen free). All procedures were conducted using aseptic techniques under isoflurane anaesthesia (2%, Abbott Laboratories, UK) in 2l/min O_2. Surgical anaesthesia was assessed by loss of the paw pinch reflex. Buprenorphine was administered intramuscularly (50 µg/kg, *i.m.*, Vetergesic, Alstoe Animal Health)

for pain relief. All animals were singly housed and allowed to recover for 7-10 days before recording baseline blood pressure for three 3 nights and 2 days in a quiet room. Blood pressure, heart rate activity was monitored at 10 min intervals, for duration of 2 min using the DSI software (DSI Dataquest A.R.T.) and data analysed in Microsoft Excel and GraphPad Prism 5. Data are represented as an average of 6h measurement.

Angiotensin-II murine hypertension model

Following baseline blood pressure measurement using radiotelemetry, C57BL/6J mice were implanted *s.c.* in the mid-scapular region under isoflurane anaesthesia, with osmotic pump (1002; Alzet) containing Angiotensin II (AngII, Sigma) at a dose of 1.1mg/kg/day or saline (control) for 14 days, as previously described.[7, 10] Blood pressure measurement using radiotelemetry was resumed. Mice were treated daily with vehicle (0.219M Mannitol, 5% HPCD, 1.6% ammonium acetate at pH 6.5) or αAnalogue[6] (50nmol/kg, *s.c.*, Novo Nordisk) at different time-points, as detailed below.

Experimental design 1: Animals were divided into four groups: vehicle (saline), vehicle (AngII), αAnalogue (saline) and αAnalogue (AngII). Following, saline or AngII osmotic pump infusion, mice received daily treatment of vehicle or αAnalogue (50nmol/kg, *s.c.,* Novo Nordisk) at Day 1 to Day 14 of AngII infusion (Figure 2). Total food intake (g), water consumption (ml) and body weight (g) was recorded throughout the study.

Experimental design 2: Animals were divided into two groups: vehicle (AngII) and αAnalogue (AngII). All mice received osmotic pumps containing AngII (1.1mg/kg/day) and at day 7 following AngII infusion when mice showed a significant increase in blood pressure from baseline, they received daily treatment of vehicle or αAnalogue (50nmol/kg, *s.c.,* Novo Nordisk) at Day 7 to Day 14 of AngII infusion (Figure 6).

At the end of the study (Day 15), mice were briefly anaesthetised with isoflurane (Abbott Laboratories) in 2l/min O_2 and blood was collected via cardiac puncture. Mice were culled by cervical dislocation and organs (heart and aorta) were harvested for post-analysis. Heart, lung, kidney and spleen from each mouse was excised, dried on filter paper and weighed. Organs' weights were normalised to body weight and tibia length.

Cardiac hypertrophy murine model

Mice were surgically subjected to pressure-overload induced-cardiac hypertrophy and heart failure as previously described.[11] Briefly, suprarenal aortic constriction was performed in anesthetised mice (2% isoflurane carried in O_2, Abbott Laboratories), where the abdominal aorta was ligated to the width of a 28G needle (0.36mm) using suture thread (8-0, Ethilon Sutures, Ethicon) in between the superior mesenteric and celiac bifurcation. Sham surgeries were performed in the same manner, excluding the

constriction. Post-surgery analgesia was provided in the form of buprenorphine (50 µg/kg, *i.m.*, Vetergesic). Following surgery, mice were treated with daily with vehicle (0.219M Mannitol, 5% HPCD, 1.6% ammonium acetate at pH 6.5) or αAnalogue[6] (50nmol/kg, *s.c.*, Novo Nordisk) for 5 weeks. Body weight, food and water intake were measured throughout the study. At the end of week 5, mice were briefly anaesthetised with isoflurane (Abbott Laboratories) in in 2l/min O_2 and blood was collected via cardiac puncture. Mice were culled by cervical dislocation and organs were harvested for post-analysis. Heart, lung, kidney and spleen from each mouse was excised and weighed. Organs' weights were normalised to body weight and tibia length.

Echocardiography

Anesthesia in mice was induced in a chamber with 5% isoflurane for 1 min, afterwards maintained with 1.5% on a heated platform. Imaging was performed 10 min after induction using a Vevo 2100 Imaging System with a 40-MHz linear probe (Visualsonics, Canada), as previously described.[11, 12] Cardiac dimensions as well as systolic function were assessed using M-mode imaging in long axis views. The relative wall thickness (RWT) in diastole was calculated as follows: RWT = (septal wall thickness + posterior wall thickness) / left ventricular diameter. Data analysis was performed with Vevo®2100 software v.1.2.1 (Visualsonics).

Light aversion assay

The light aversion assay is based on rodents' natural preference for darker areas compared to lighter areas[13, 14] and was used to study photophobia in rodents, a common symptom of migraine.[15] The apparatus consisted of two equal compartments: one-half (20 (d) x 20 (w) x 14 (h) cm) black plexiglass, with a black lid on top, the remaining half was transparent to enable behavioural assessment and recording. Mice were able to move freely between the two compartments through a small doorway (5 × 5 cm) in the center of the black plexiglass wall separating the two compartments, as described previously.[14] Lighting conditions (1000 lux) were established with the aid of 100-watt incandescent lamp, placed 45 cm above the centre of the box floor to the test arena and confirmed with a luxmeter (Mastech Light Meter, LX1010B, Mestech UK). At 1000 lux, light intensities in the dark zone were about 200 lux immediately inside the opening, 30 lux at the back wall across from the opening, and 10 lux at all four corners.

Mice were trained in the light/dark box under a range of lighting conditions (500-1000 lux) twice for 7 days. Mice that displayed equal preference between light/dark compartments (>40% light preference) were used in our study. Following acclimation in the testing room for 1h (200 lux), baseline recordings (10 min) were obtained, followed by systemic administration of vehicle (0.219M Mannitol, 5% HPCD, 1.6% ammonium acetate at pH 6.5) or αAnalogue (50 nmol/kg, *s.c.*) or the positive control glyceryl trinitrate (GTN, 320 nmol/kg, *i.v.*). Behavioural responses were resumed for 10min at 2h following

treatment.[16]

To evaluate the effects of chronic administration of the αAnalogue (5 weeks), sham mice were assessed on week 5 for light aversion at 1h following systemic administration of vehicle (0.219M Mannitol, 5% HPCD, 1.6% ammonium acetate at pH 6.5) or αAnalogue (50 nmol/kg, *s.c.*); behavioural responses were recorded for 10min.

Mouse behaviour was digitally recorded using an IPEVO camera (IPEVO, UK), placed 30cm above the light/dark box and attached to a laptop. Videos were continuously recorded at 30 frames/s and decomposed to individual frames of 1s interval. Each 10min experimental interval was taken to start immediately after the mouse entered the dark chamber. Behavioural experiments were conducted by a blinded observer and analysis was conducted procedurally with a custom ImageJ script, applying foreground extraction to determine the presence of the mouse against the white background of the light box floor across sequential frames. Absolute duration and % dwell time within the light and dark chambers was automatically calculated, as was the number of transitions, interpreted as crossings. Data was represented as the % of time spent in light during the 600s test session.

Glucose tolerance test

Glucose tolerance tests (GTT) were performed following a 6h fast (from 08:30 to 14:30), as previously described.[17] Glucose (1g/kg, Sigma) was injected *i.p.* and blood glucose levels were monitored for 2h (from 08:30 to 14:30) at the indicated time points after injection with a One Touch Vita glucose meter (Lifescan, UK). GTT was determined at baseline and at Day 14 following daily treatment with the αAnalogue (50nmol/kg, *s.c.*) or its respective vehicle (0.219M Mannitol, 5% HPCD, 1.6% ammonium acetate at pH 6.5).

Core body temperature measurement using radiotelemetry

Core body temperature and activity were measured using radio-telemetry as described previously.[18] Briefly, animals were anaesthetised by isoflurane (2%, Abbott Laboratories, UK) in 2l/min O_2, placed on a homeothermic heating pad and buprenorphine (50µg/kg, *i.m.*, Vetergesic, Alstoe, Animal Health, UK) was administered peri-operatively. The abdomen was shaved and scrubbed using surgical scrub and a small ventral midline abdominal incision (< 2 cm) was made to expose the abdominal muscle wall. A ventral incision was made on the abdominal wall and a small volume of (< 80 µl) sterile saline was applied to facilitate the insertion of the radio telemetry transmitter (TA10TA-F10; DSI, St Paul, Minnesota, USA). Following implantation, the abdominal wall and skin were sutured separately using absorbable sutures (Vicryl ® 4.0, Ethicon, Johnson & Johnson, UK). The mice were monitored until ambulatory and were individually housed with food and water *ad libitum*. Mice were weighed and examined daily, and were allowed a minimum 7 days post-surgical recovery period. Cages containing the telemetered animals were placed on the receiver plates (RPC-1; Data Sciences Incorporated (DSI,

Minnesota, USA); radio signals from the implanted transmitters were monitored via a fully automated data acquisition system (DataquestART, version 3.1; DSI, Minnesota, USA). Locomotor activity and core body temperature were monitored at 10 min intervals, for duration of 2 min.

Following baseline measurement for 1h, the αAnalogue (50nmol/kg, *s.c.*) and its respective vehicle (0.219M Mannitol, 5% HPCD, 1.6% ammonium acetate at pH 6.5) were administered *s.c.* and measurements were recorded for 24h thereafter.

Measurement of gene expression using Real-time reverse transcription polymerase chain reaction

Real-time reverse transcription polymerase chain reaction (RT-qPCR) was carried out as previously described.[3, 7, 10]

Total ribonucleic acid (RNA) was extracted from aorta, heart and kidney tissue using the Qiagen RNeasy Microarray tissue mini Kit (Qiagen), according to manufacturer's instructions. Total RNA (1µg) was then reverse transcribed into cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Life technologies Ltd) as per manufacturer's instructions. A thermal cycler (DNA Engine Tetrad 2 Peltier Thermal Cycler) was used for reverse transcription to complementary DNA (cDNA). Negative RT samples were carried out as a control to exclude possible contamination of genomic DNA. cDNA was then diluted 1:40 in nuclease-free water for RT-qPCR. Quantitative PCR (qPCR) was then conducted using a SYBR-green-based PCR mix (Sensi-Mix, SYBR-green No ROX; Bioline) and primers from the specific gene of interest pipetted into 100-well gene disks (Qiagen) by an automated robot (CAS1200; Qiagen Corbett), followed by PCR in a Corbett Rotor-gene 6000. Settings were as follows; initial denaturation: 10min at 95°C; cycling: 45 cycles- 10 s at 95°C, 15 s at 57°C, and 5s at 72°C; melt: 68-90°C. Samples were subjected to melting curve analysis to confirm amplification specificity. Data were collected as copies/µl and normalised against murine hypoxanthine phosphor-ribosyltransferase (HPRT), β-2-microglobulin (B_2M) and β-actin expression using GeNorm3.4 software. A list of primers are summarised in Supplemental Table I.

Western blotting

Western blot analysis was performed as previously described.[1, 2, 7] Briefly, aorta, mesentery, heart and kidney tissue was homogenised in SDS lysis buffer containing protease inhibitor (1tablet/50ml, Roche, Germany) and protein concentrations were determined. Samples were loaded onto a 7-12% Tris SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes using a semi-dry technique (Bio-Rad). After blocking with 5% non-fat dry milk, membranes were incubated with a primary antibody (4°C, overnight), and incubated with the secondary antibody at room temperature for 1h. Proteins were detected by enhanced chemiluminescence (ECL, Piercenet) and developed using the Syngene gel doc dark room system. Densitometric analysis was performed using Image J analysis software (NIH, USA). Antibodies against eNOS (1:500, Ab5589), HO-1 (1:500,

Ab13248), nitrotyrosine (1:500, Ab61392)[2], GPX-1 (1:1000, Ab22604), CLR (1:500, Ab173562) and RAMP1 (1:1000, Ab156575) were purchased from Abcam. Antibodies against NOX-2 (gp91phox, 1:500, 611414) were obtained from BD Transduction, α-SMA (1:1000, A2547) from Sigma and klotho (1:500, KM2076) from Cosmo Bio Co. Ltd. p-p38 (1:1000, 9216) and total-p38 (1:1000, 9212) were purchased from Cell Signalling. GAPDH (1:4000, AM4300) was purchased from Life Technologies Ltd and used for a loading control. Note that for nitrosylated proteins detected using anti-nitrotyrosine antibody, densitometric analysis was conducted for the entire lane and normalised to GAPDH.

Quantification of noradrenaline using ELISA

Plasma and renal noradrenaline (NA) content were quantified using a commercially available NA ELISA kit (RE59261; IBL International, Hamburg, Germany), as previously described.[1] Briefly, kidneys were homogenised in phosphate buffered saline (1M), supplemented with protease inhibitors (Roche, Germany). Lysates were cleared by centrifugation at 2,600 *g* for 15 min at 4°C. NA content was extracted from both plasma and kidney tissue lysate and assessed by standard ELISA technique, according to the manufacturer instructions. The limit of sensitivity was 20 pg/ml and the linearity limit was 8.0 ng/ml. Cross-reactivity to other catecholamines or metabolites was manufacturer tested as <0.02%. Protein concentration of each kidney samples were determined by Bradford dye-binding method (Bio-rad) and noradrenaline content was normalised to mg of protein.

Quantification of IL-6 and TNF-α using ELISA

Briefly, frozen kidney tissues were homogenised in phosphate buffered saline (1M), supplemented with protease inhibitors (Roche, Germany). Lysates were cleared by centrifugation at 2,600 *g* for 15 min at 4°C. Renal interleukin-6 (IL-6) and Tumour necrosis factor-α (TNF-α) content were quantified using a commercially available ELISA kit (RAB0308 & RAB0477, Sigma, UK), following manufacturer's protocol. Protein concentration of each kidney samples were determined by Bradford dye-binding method (Bio-rad). The final concentrations of both IL-6 and TNF-α were calculated with respect to the total amount of protein (mg).

Histology

Aorta, heart and kidney tissues were fixed in 4% paraformaldehyde (overnight, 4°C), as previously described.[7, 19, 20] For heart and kidney samples, after routine paraffin wax embedding, transverse sections (6μm) were prepared using a Reichert-Jung 2030 Biocut microtome onto poly-L-lysine slides. For aorta samples, following fixation aorta segments were embedded in optimal cutting temperature compound (OCT, VWR), frozen on dry ice in a cryomould and cryosectioned on a microtome cryostat (6μm, Bright, UK) on Superfrost Plus glass slides. Sections were allowed to dry overnight. Approximately 8-10 transverse sections were obtained from each heart or aorta per mouse for staining.

Aorta Staining: Sections of aorta were stained with Masson's trichrome, as previously described[7, 21]. All slides were imaged using an Olympus Colourview III camera, connected to an Olympus BX51 microscope (x10 magnification). Images were taken using CellSens Dimension viewing software (Version 1.1.3, Olympus). Aortic wall width (medial thickness) were measured (mean of 8 measurements taken from at least 3 sections for each 4 mice/group) using ImageJ analysis software (scale bars, 100μm; NIH, USA).

Heart Staining: Picrosirius Red (0.1% w/v) staining was used to visualise collagen fibres in the heart[20] and fibrosis was assessed blindly. All slides were imaged using a Leica Diaplan microsope (x10 magnification) and images captured using ProgRes Capture Pro viewing software (Version 2.8.8, JENOPTIK) under bright light and circular polarised light according to a modified Junqueira method.[22, 23] Cardiac fibrosis was quantified on circular polarised light images using Image J analysis software (scale bars, 200μm; NIH, USA).

Conjugated wheat germ agglutinin (WGA, CF488A, Biotium) staining was applied to outline cardiomyocyte boundaries and quantify cross-sectional area.[20] All slides were imaged using an Olympus Colourview III camera, connected to an Olympus BX51 microscope (x40 magnification). Images were taken using CellSens Dimension viewing software (Version 1.1.3, Olympus). Myocyte cross-sectional area was employed as an index of cardiac hypertrophy and determined on fibres with circular shapes in a blinded fashion by quantitative image analysis using Image J (scale bars, 20μm; NIH, USA).

Capillaries were immunostained with isolectin B4 (Vector B-1205) and capillary density was quantified as the number of capillaries per mm^2 of LV sections[11]. All slides were imaged using an Olympus Colourview III camera, connected to an Olympus BX51 microscope (x40 magnification). Images were taken using CellSens Dimension viewing software (Version 1.1.3, Olympus). Number of capillaries were manually counted in a blinded fashion by quantitative image analysis using Image J (scale bars, 20μm; NIH, USA).

Apoptosis was detected by using terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) staining (Millipore S7111 kit) in LV sections[24]. All slides were imaged using an Olympus Colourview III camera, connected to an Olympus BX51 microscope (x20 magnification). Images were taken using CellSens Dimension viewing software (Version 1.1.3, Olympus). TUNEL-positive myocytes and DAPI-stained nuclei were manually counted by quantitative image analysis using Image J (scale bars, 50μm; NIH, USA).

Kidney Staining: Sections of kidney were stained with Periodic acid–Schiff's (PAS) and counter stained with hematoxylin, as previously described[25]. All slides were imaged using an Olympus Colourview III camera, connected to an Olympus BX51 microscope (x40 magnification). Images were taken using

CellSens Dimension viewing software (Version 1.1.3, Olympus). A semi quantitative system was used to grade mesangial expansion by either diffuse (irregular streaks) or segmental (localized to a lobule) type. In each of the experimental animals, 20 glomeruli were examined for mesangial expansion and each glomerulus was graded 0 to 4 corresponding to the percentage of mesangial matrix covering/occupying the urinary space of the Bowman's capsule. Glomeruli were assessed and graded 1-4, as follows: grade 1, involvement of 0-24%; grade 2, 25-29%; grade 3, 50-74% or grade 4; 75-100% of mesangial area to Bowman's capsule (scale bars, 20μm; NIH, USA). Mean glomerular score index was determined for each kidney[26].

All morphometric measurements were performed in a blinded manner.

Statistical analysis

Data in the manuscript is expressed as mean $\pm$ SEM. Statistical analysis was performed using a two-tailed Student's *t*-test (2 unpaired groups), one-way or repeated measures two-way analysis of variance (ANOVA) followed by Bonferroni's comparison *post hoc* test (multiple groups comparison). $p<0.05$ was considered to represent a significant difference.

Supplemental Tables

Supplemental Table 1. Gene primer sequences

Target Gene	Primer Sequence	Accession number
Akt	F: CGTCGCCAAGGATGAGGTTG R: GTCGTGGGTCTGGAATGAGT	NM_001165894.1
α-MHC	F: GCTGGAAGAAAAGCTCAAGAAGAAA R: TCTCTATCTGCACGGATGTGG	NM_001164171.1
β-MHC	F: CACCTACCAGACAGAGGAAGA R: GGAGCTGGGTAGCACAAGA	NM_080728.2
α-skeletal actin	F: CTAAATCCAAGTCCTGCAAGTG R: ACATGGTGTCTAGTTTCAGAGG	NM_001272041.1
ANP	F: GGATTTCAAGAACCTGCTAGACC R: GCAGAGCCCTCAGTTTGCT	NM_008725.3
B_2M	F: GTCGCTTCAGTCGTCAGCA R: TTGAGGGGTTTTCTGGATAGCA	NM_009735.3
Bcl-2	F: AGGCTGGGATGCCTTTGTGG R: TGTTTGGGGCAGGTTTGTCG	NM_009741.5
BNP	F: TGGGCTGTAACGCACTGAA R: TGTTGTGGCAAGTTTGTGCTT	NM_001287348.1
Collagen Type 1 α1	F: TCTGACTGGAAGAGCGGAGAG R: AGACGGCTGAGTAGGGAACA	NM_007742.4
Collagen Type 1 α2	F: TGGATACGCGGACTCTGTTG R: CCCTTTCGTACTGATCCCGATT	NM_007743.2
Collagen Type 3 α1	F: GGGAGGAATGGGTGGCTATC R: CTGGGCCTTTGATACCTGGA	NM_009930.2
Collagen Type 4 α1	F: CTGGAGAAAAGGGCCAGAT R: TCCTTAACTTGTGCCTGTCC	NM_009931.2
CTGF	F: GGGCCTCTTCTGCGATTC R: ATCCAGGCAAGTGCATTGGTA	NM_010217.2
Cystatin C	F: GAGTACAACAAGGGCAGCAAC R: AGCAGAGTGCCTTCCTCATCA	NM_009976.4
eNOS	F: GACCCTCACCGCTACAA AT R: GTCCTGGTGTCCAGATCCAT	NM_008713.4
Fibronectin	F: CCGGTGGCTGTCAGTCAGA R: CCGTTCCCACTGCTGATTTATC	NM_006495700.2
GPX-1	F: TTCGGACACCAGGAGAATGG R: TAAAGAGCGGGTGAGCCTTC	NM_008160.6
HIF-1α	F: CGAGAACGAGAAGAAAAAGATGAGT R: CGTAAATAACTGATGGTGAGCCT	NM_010431.2
HO-1	F: CAACATTGAGCTGTTTGAGGAG R: CTCTGACCAAGTGACGCCAT	NM_010442.2
	F: CCTGGTTCATCATCGCTAATC	NM_013556.2

HPRT	R: TCCTCCTCAGACCGCTTTT	
MMP-2	F: GACAAGTTCTGGAGATACAATGAAGTG R: CAGGTTATCAGGGATGGCATTC	NM_006530751.1
NGAL	F: AATGTCACCTCCATCCTGGTCA R: GACAGCTCCTTGGTTCTTCCATAC	NM_008491.1
NF-κB	F: CCTACGGAACTGGGCAAATGT R: TCCCCTCTGTTTTGGTTGCT	NM_ 008689.2
NOX-2	F: ACTCCTTGGGTCAGCACTGG R: GTTCCTGTCCAGTTGTCTTCG	NM_007807.5
NQO1	F: TCATTCTCTGGCCGATTCA R: TGCTGTAAACCAGTTGAGGTTC	NM_008706.5
Osteopontin	F: AAACCAGCCAAGGTAAGCCT R: GCAAAAGCAATCACTGCCA	NM_001204201.1
p53	F: ATGCCCATGCTACAGAGGAG R: AGACTGGCCCTTCTTGGTCT	NM_001127233.1
RANTES	F: TGCTCCAATCTTGCAGTCGT R: GCGTATACAGGGTCAGAATCAAG	NM_013653.3
SERCA-2	F: TGGAACCTTTGCCGCTCATTT R: CAGAGGCTGGTAGATGTGTT	NM_009722.3
TGF-β_1	F: TCAGACATTCGGGAAGCAGT R: GCCCTGTATTCCGTCTCCTTG	NM_011577.2
TIMP-2	F: GATTCAGTATGAGATCAAGCAGATAAAGA R: GCGAGACCCCGCACACT	NM_011594.3
α-SMA	F: ACTACTGCCGAGCGTGA R: ATAGGTGGTTTCGTGGATGC	NM_007392.3
β-actin	F: CACAGCTTCTTTGCAGCTCCTT R: TCAGGATACCTCTCTTGCTCT	NM_007393.5

Supplemental Table 1. List of gene primer sequences used for qRT-PCR studies.

Supplemental Table 2: Changes in organ weight in AngII and saline-infused mice pre-treated with αAnalogue or vehicle

Organs	Vehicle		αAnalogue	
	Saline	AngII	Saline	AngII
Total Heart:Body Weight (mg/g)	4.4 ± 0.3	6.3 ± 0.2 ***	4.5 ± 0.2	5.2 ± 0.2 ††
Total Heart:Tibia length (mg/mm)	7.9 ± 0.4	10.0 ± 0.4 ***	7.5 ± 0.2	8.4 ± 0.3 ††
Left Ventricle:Body Weight (mg/g)	3.4 ± 0.2	4.9 ± 0.1 ***	3.5 ± 0.2	4.2 ± 0.1 †††
Lung Oedema (Wet:Dry Ratio)	4.2 ± 0.1	4.2 + 0.1	4.1 ± 0.1	4.1 + 0.1
Kidney:Body Weight (mg/g)	5.8 ± 0.3	5.8 ± 0.3	5.8 ± 0.3	5.7 ± 0.2
Kidney:Tibia length (mg/mm)	10.8 ± 0.3	9.1 ± 0.4	9.4 ± 0.2	9.4 ± 0.6
Spleen:Body Weight (mg/g)	2.5 ± 0.2	3.1 ± 0.2	2.7 ± 0.2	3.1 ± 0.3
Spleen:Tibia length (mg/mm)	4.7 ± 0.2	4.8 ± 0.3	4.5 ± 0.3	5.1 ± 0.4

Supplemental Table 2. Changes in organ weight in the heart of AngII and saline-infused mice treated daily with α-CGRP analogue (αAnalogue, 50nmol/kg) or vehicle (*s.c.*) daily for 14 days. Mice were implanted with osmotic pumps containing saline or AngII (1.1mg/kg/day) and treated daily with vehicle or αAnalogue (50nmol/kg, *s.c.*) for 14 days (n=6-9). At day 15, mice were sacrificed, organs were weighed and normalised to body weight (mg/g) or tibia length (mg/mm). Results show mean ± SEM. ***p<0.001 vs vehicle-treated saline-infused mice; ††p<0.01, †††p<0.001 vs vehicle-treated AngII-infused mice (2-way ANOVA + Bonferroni *post hoc* test).

Supplemental Table 3: Changes in gene expression in the heart of AngII and saline-infused mice pre-treated with αAnalogue or vehicle

	Vehicle		αAnalogue	
Gene	**Saline**	**AngII**	**Saline**	**AngII**
CTGF	89.3 ± 14.6	220.3 ± 44.0 *	152.0 ± 42.9	67.6 ± 20.1 †
Akt	499.1 ± 80.1	721.8 ± 96.6	666.7 ± 78.6	365.7 ± 59.3 †
TIMP-2	337.6 ± 22.5	396.2 ± 33.5	391.5 ± 52.1	223.5 ± 30.0 *, ††
NF-κB	231.6 ± 16.5	263.6 ± 25.2	276.9 ± 14.6	185.3 ± 12.7 **, †
Bcl-2	16.0 ± 1.5	17.3 ± 2.0	13.5 ± 2.1	12.7 ± 1.7
p53	5082 ± 569.4	7267 ± 543.1 *	6475 ± 518.5	4926 ± 359.5 ††

Supplemental Table 3. Changes in gene expression in in the heart of AngII and saline-infused mice treated daily with α-CGRP analogue (αAnalogue, 50nmol/kg) or vehicle (*s.c.*) daily for 14 days. Mice implanted with osmotic pumps containing saline (S) or AngII (A, 1.1mg/kg/day) and treated daily with vehicle or αAnalogue (50nmol/kg, s.c.) for 14 days. mRNA expression measured by qRT-PCR (n=5-11) for connective tissue growth factor (CTG), Protein Kinase B (PKB, Akt), tissue inhibitor of metalloproteinase-2 (TIMP-2), nuclear factor kappa B cells (NF-κB), apoptosis regulator B-cell lymphoma 2 (Bcl-2) and apoptotic marker p53 in the mice heart. Results expressed as copy numbers per µl normalised to HPRT, B_2M and β-actin. Results show mean ± SEM. *$p<0.05$ vs vehicle-treated saline-infused mice; †$p<0.05$, ††$p<0.01$ vs vehicle-treated AngII-infused mice (2-way ANOVA + Bonferroni *post hoc* test).

Supplemental Table 4. Changes in gene expression in the aorta of vehicle and αAnalogue treated AngII-infused mice

	AngII	
Gene	**Vehicle**	**αAnalogue**
Vascular dysfunction		
eNOS	210.2 ± 14.4	162.2 ± 31.5
Akt	663.3 ± 23.6	567.9 ± 30.1 *
Remodelling and fibrosis		
TGF-β	3282.2 ± 395.3	1567.4 ± 412.6 *
CTGF	2957.2 ± 270.3	1513.3 ± 412.6 *
COL1*A1*	8869.7 ± 2537.2	5253.2 ± 1553.8
COL3*A1*	2765.6 ± 802.5	1139.5 ± 325.45
α-SMA	5288.1 ± 343.5	3272.2 ± 271.6 **
Vascular Inflammation		
RANTES	2524.6 ± 1132.0	928.9 ± 228.8
Osteopontin	36.0 ± 10.9	4.8 ± 2.4 *
Oxidative Stress		
HO-1	1169.0 ± 349.0	226.3 ±68.2 *
NOX-2	52.8 ± 1.4	32.3 ± 2.8 **

Supplemental Table 4. Changes in gene expression in the aorta of vehicle and α-CGRP analogue (αAnalogue) treated Angiotensin II (AngII)-infused mice. Mice infused with AngII (1.1mg/kg/day) and treated daily with vehicle or αAnalogue (50nmol/kg, *s.c.*) at day 7 to 14. mRNA expression measured by qRT-PCR (n=4) for endothelial nitric oxide (eNOS), Protein kinase B (PKB, Akt), transforming growth factor beta-1 (TGF-β1), connective tissue growth factor (CTGF), collagen type 1 α1 (COL*1A1*), collagen type 3 α1 (COL*3A1*), alpha-smooth muscle actin (α-SMA), RANTES, osteopontin, haem-oxygenase 1 (HO-1), NAD(P)H dehydrogenase, quinone-1 (NQO1) and NADPH oxidase-2 (NOX-2) in the mice aorta. Results expressed as copy numbers per µl normalised to HPRT, B2M and β-actin. Results are shown as mean ± SEM. *p<0.05, **p<0.01 vs vehicle-treated AngII-infused mice (two-tailed Student *t*-test).

Supplemental Table 5: Changes in organ weight in αAnalogue or vehicle-treated AngII-induced hypertensive mice		
Organs	**AngII**	
	Vehicle	**αAnalogue**
Total Heart:Body Weight (mg/g)	6.3 ± 0.5	4.9 ± 0.2 *
Total Heart:Tibia length (mg/mm)	9.4 ± 0.4	7.5 ± 0.6 *
Left Ventricle:Body Weight (mg/g)	5.2 ± 0.4	3.9 ± 0.1 *
Lung Oedema (Wet:Dry Ratio)	3.7 ± 0.2	3.9 ± 0.3
Kidney:Body Weight (mg/g)	5.8 ± 0.3	5.3 ± 0.3
Kidney:Tibia length (mg/mm)	8.4 ± 0.2	8.2 ± 0.1
Spleen:Body Weight (mg/g)	2.6 ± 0.3	2.9 ± 0.2
Spleen:Tibia length (mg/mm)	3.8 ± 0.3	4.5 ± 0.6

Supplemental Table 5. Changes in organ weight in the heart of α-CGRP analogue (αAnalogue, 50nmol/kg) or vehicle (*s.c.*)-treated AngII-infused mice. Mice were infused with AngII (1.1mg/kg/day) osmotic pumps for 14 days and treated with vehicle or αAnalogue (50nmol/kg) on Day 7-14 (n=4). At day 15, mice were sacrificed, organs were weighed and normalised to body weight (mg/g) or tibia length (mg/mm). Results show mean ± SEM. *$p<0.05$ vs vehicle-treated AngII-infused mice (two-tailed Student t-test).

Supplemental Table 6: Changes in gene expression in the heart of vehicle and αAnalogue treated AngII-infused mice		
	AngII	
Gene	**Vehicle**	**αAnalogue**
Remodelling and fibrosis		
CTGF	1118 ± 85.6	719.0 ± 206.3
Fibronectin	586.0 ± 62.0	330.6 ± 87.6
COL1*A1*	825.5 ± 140.0	569.3 ± 157.8
COL3*A1*	852.2 ± 140.0	282.5 ± 91.9 *
COL4*A1*	4670.9 ± 415.7	4108.7 ± 512.0
SERCA-2	189.2 ± 13.5	163.7 ± 18.7
ANP	40224 ± 7485.1	6026.7 ± 2559.2 **
BNP	1365.6 ± 220.9	1517.8 ± 641.2
Oxidative Stress		
NOX-2	67.7 ± 7.1	44.4 ± 3.9 *

Supplemental Table 6. Changes in gene expression in the heart of vehicle and α-CGRP analogue (αAnalogue) treated Angiotensin II (AngII)-infused mice. Mice infused with AngII (1.1mg/kg/day) and treated daily with vehicle or αAnalogue (50nmol/kg, s.c.) at day 7 to 14 (n=4). mRNA expression measured by qRT-PCR for connective tissue growth factor (CTGF), fibronectin, collagen type 1 α1 (COL*1A1*), collagen type 3 α1 (COL*3A1*), collagen type 4 α1 (COL*4A1*), sarcoplasmic reticulum Ca2+ ATPase-2 (SERCA-2), atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and NADPH oxidase-2 (NOX-2) in heart. Results expressed as copy numbers per µl normalised to HPRT, B2M and β-actin. Data represent mean + S.E.M. *$p<0.05$, **$p<0.01$ vs vehicle-treated AngII-infused (two-tailed Student t-test).

Supplemental Table 7. Echocardiographic parameters of left ventricular size and function 5 weeks post AAC-induced hypertrophy and heart failure in mice treated with αAnalogue or vehicle

Parameters	Vehicle		αAnalogue	
	Sham	AAC	Sham	AAC
n	8	6	8	8
HR [bpm]	430.3 ± 11.59	455.5 ± 36.49	452.9 ± 10.37	457.9 ± 17.49
LVID;d [mm]	4.18 ± 0.10	4.36 ± 0.13	4.10 ± 0.11	4.32 ± 0.07
LVID;s [mm]	3.16 ± 0.08	3.68 ± 0.21*	3.01 ± 0.09	3.32 ± 0.09
LVV;d [µl]	40.14 + 2.43	59.04 + 7.82**	35.76 + 2.80	45.13 + 3.04
LVV;s [µl]	78.41 ± 4.39	86.44 ± 5.97	75.00 ± 4.98	84.61 ± 3.16
septW [mm]	0.78 + 0.03	1.02 + 0.04 ***	0.79 + 0.03	0.87 + 0.03 ††
postW [mm]	0.74 + 0.03	1.03 + 0.06 ***	0.71 + 0.03	0.78 + 0.02 †††
rWT	0.37 + 0.02	0.47 + 0.02 **	0.37 + 0.02	0.38 + 0.01 ††
SV [µl]	38.27 + 2.30	27.40 + 2.7 **	39.24 + 2.43	39.48 + 1.34 ††
EF [%]	48.80 + 1.17	33.10 + 5.17 ***	52.51 + 1.24	46.87 + 1.96 ††
FS [%]	24.39 + 0.72	15.91 + 2.92 ***	26.67 + 0.79	23.37 + 1.17 ††

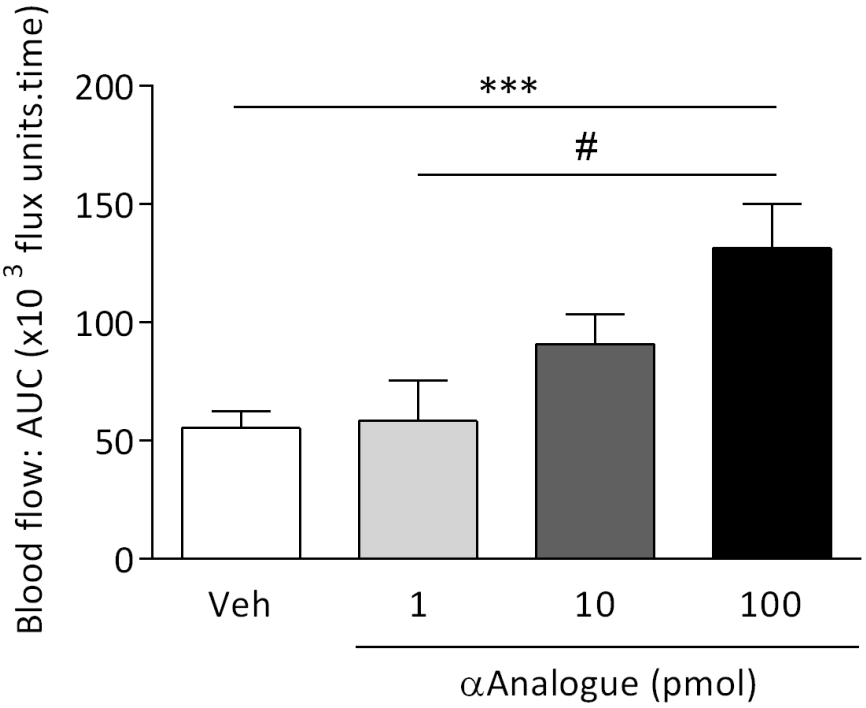
Supplemental Table 7. Echocardiographic parameters of left ventricular size and function 5 weeks post AAC-induced hypertrophy and heart failure. Mice were treated daily with vehicle or α-CGRP analogue (αAnalogue, 50nmol/kg/day, *s.c.*) post surgery (n=6-8). (HR: heart rate, LVID;d: left ventricular dimension in diastole, LVID;s: left ventricular dimension in systole, LVV;d: left ventricular volume in diastole, LVV;s: left ventricular volume in systole, septW: septal wall thickness, postW: posterior wall thickness, rWT: relative wall thickness, SV: stroke volume, EF: ejection fraction, FS: fractional shortening. Data represented as mean ± SEM (n=6-8). *p<0.05, **p<0.01, ***p<0.001 vs respective sham-treated; ††p<0.01, †††p<0.001 vs vehicle-treated AAC (2-way ANOVA + Bonferroni *post hoc* test).

Supplemental Table 8: Changes in organ weight in AAC and sham-treated WT mice pre-treated with αAnalogue or vehicle

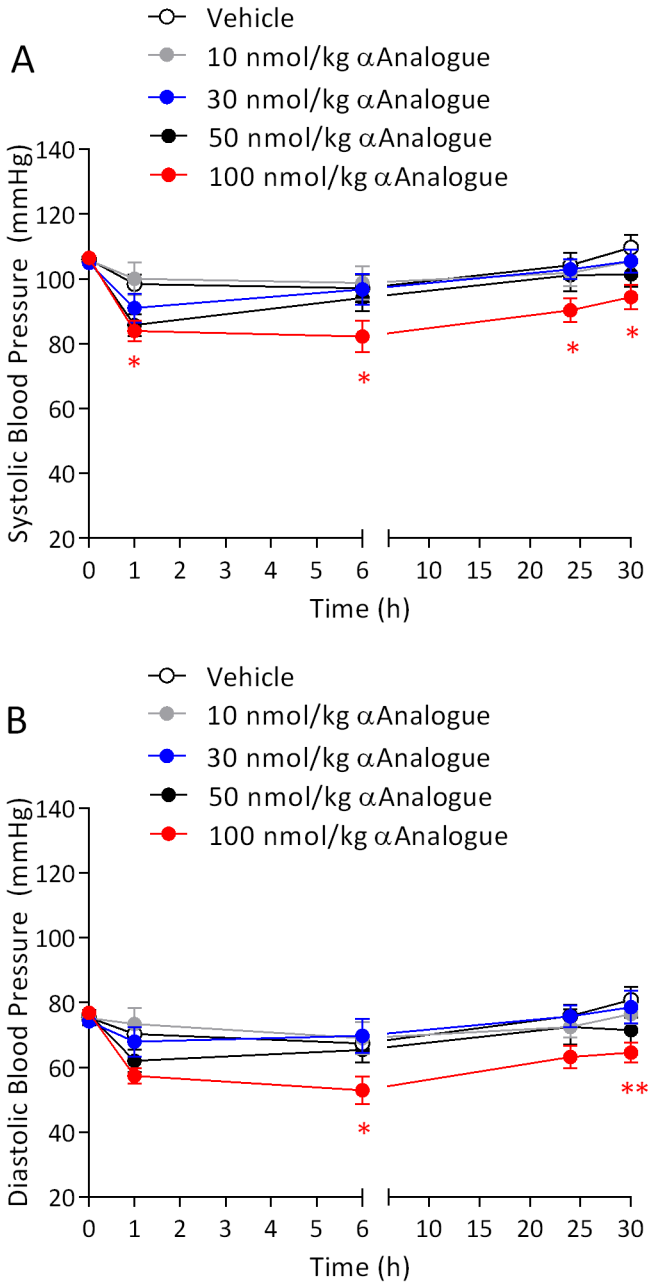
Organs	Vehicle		αAnalogue	
	Sham	AAC	Sham	AAC
Total Heart:Body Weight (mg/g)	4.7 ± 0.1	9.1 ± 0.1***	4.7 ± 0.2	5.9 ± 0.3†††
Total Heart:Tibia length (mg/mm)	7.6 ± 0.2	13.8 ± 1.2***	7.8 ± 0.3	9.6 ± 0.5†††
LV:Body Weight (mg/g)	3.5 ± 0.1	7.1 ± 0.6***	3.5 ± 0.1	4.5 ± 0.3*,†††
Lung Oedema (Wet:Dry Ratio)	4.3 ± 0.0	4.5 ± 0.3	4.2 ± 0.1	4.3 ± 0.1
Dry Lung:Tibia length (mg/g)	1.7 ± 0.1	3.8 ± 0.8***	2.0 ± 0.1	1.9 ± 0.1††

Supplemental Table 8. Changes in organ weight in α-CGRP analogue (αAnalogue, 50nmol/kg) or vehicle (*s.c.*)-treated sham or AAC-induced cardiac hypertrophy and heart failure mice. Mice were treated daily for 5 weeks post surgery. Organs were weighed and normalised to body weight (mg/g) or tibia length (mg/mm). Results show mean + SEM (n=6-8). *p<0.05, ***p<0.001 vs respective sham-treated; ††p<0.01, †††p<0.001 vs vehicle-treated AAC mice (2-WAY ANOVA + Bonferroni *post hoc* test).

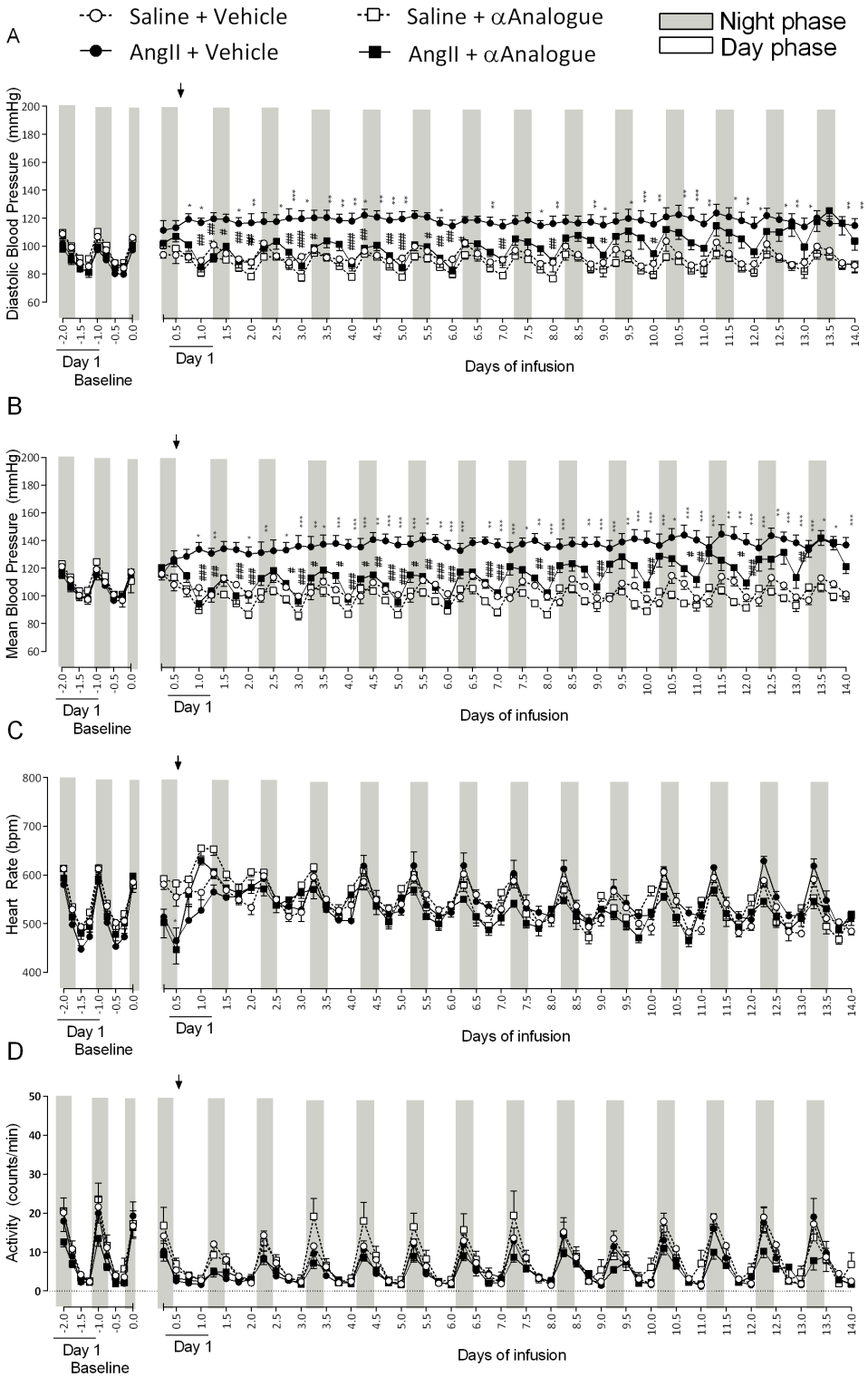
Supplemental Figures



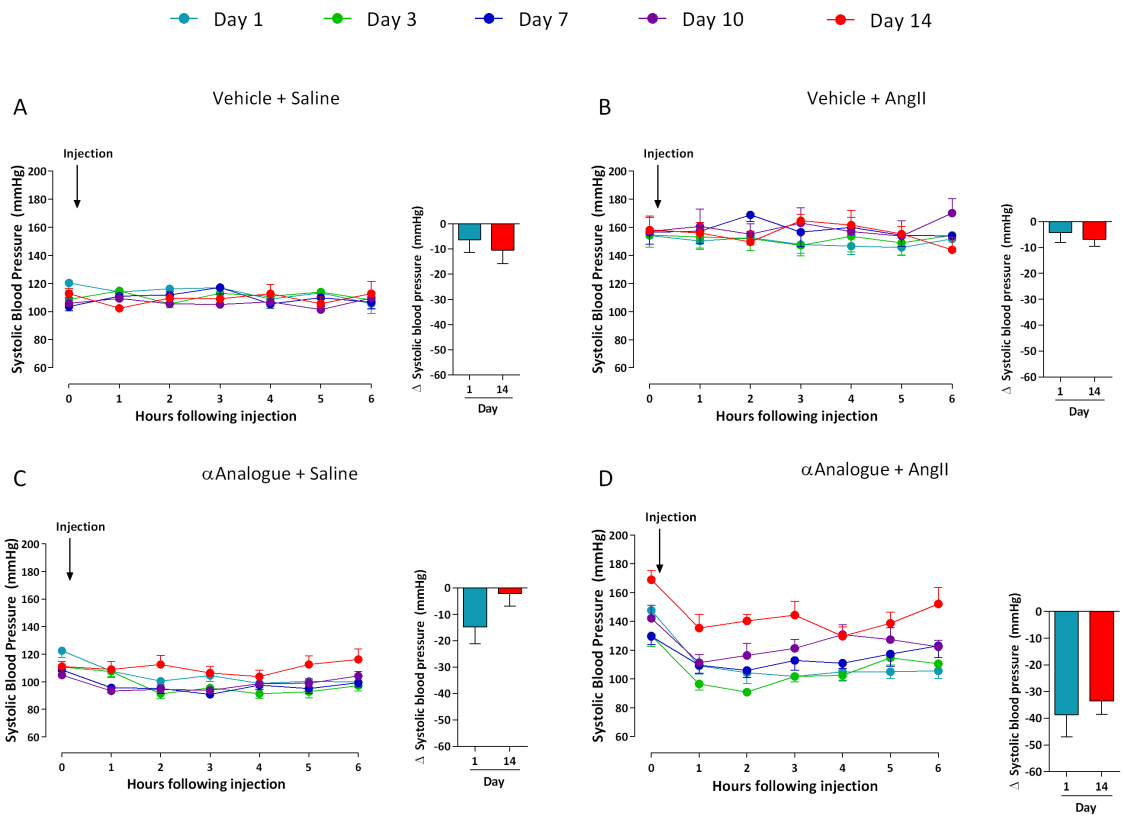
Supplemental Figure 1. Effect of local administration α-CGRP analogue (αAnalogue) on vascular skin blood flow in naïve mice. Blood flow was monitored using Full-field Laser Perfusion Imager (FLPI) in the ear skin of anaesthetised mice (n=4-6) at baseline (5 min) and following intradermal injection of αAnalogue (1-100pmol) in the ipsilateral ear and vehicle (0.219M Mannitol, 5% HPCD, 1.6% ammonium acetate at pH 6.5) in the contralateral ear. Results show mean ± SEM for area under the curve (AUC) for the 30 min recording. ***$p<0.001$ vs vehicle-treated ear; #$p<0.05$ vs αAnalogue treated ear (One-way ANOVA + Bonferroni *post hoc* test).



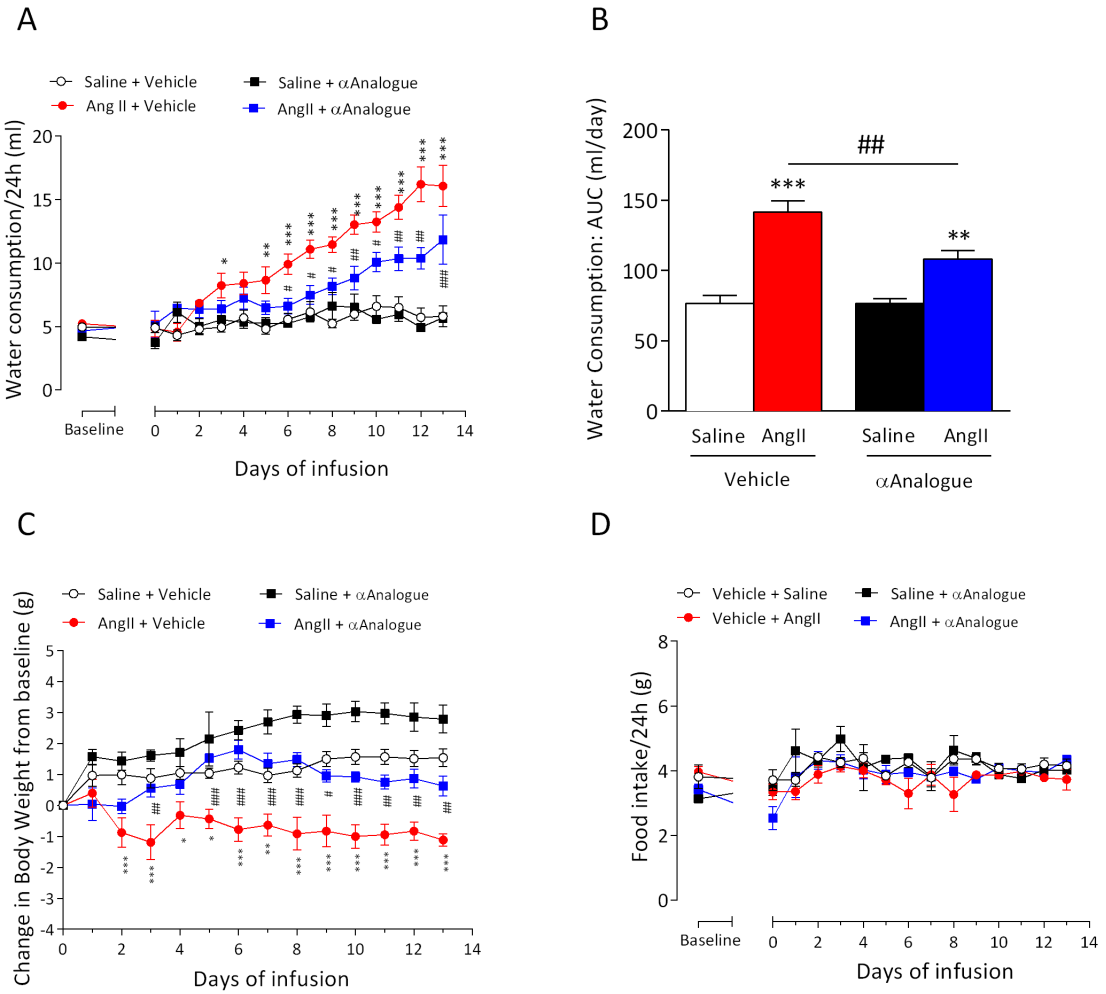
Supplemental Figure 2. Effect of systemic administration of α-CGRP analogue (αAnalogue) on blood pressure in naïve mice. (A) Systolic and **(B)** diastolic blood pressure was monitored using tail-cuff plethysmography in trained conscious restrained mice at baseline and 1, 6, 24 and 30h following vehicle (0.219M Mannitol, 5% HPCD, 1.6% ammonium acetate at pH 6.5) or αAnalogue administration (10, 30, 50 or 100nmol/kg, *s.c.*). Blood pressure values were obtained for each animal and results show mean ± SEM for each group of mice (n=7). *p<0.05, **p<0.01 vehicle vs 100nmol/kg αAnalogue treatment (Repeated measures 2-way ANOVA + Bonferroni *post hoc* test).



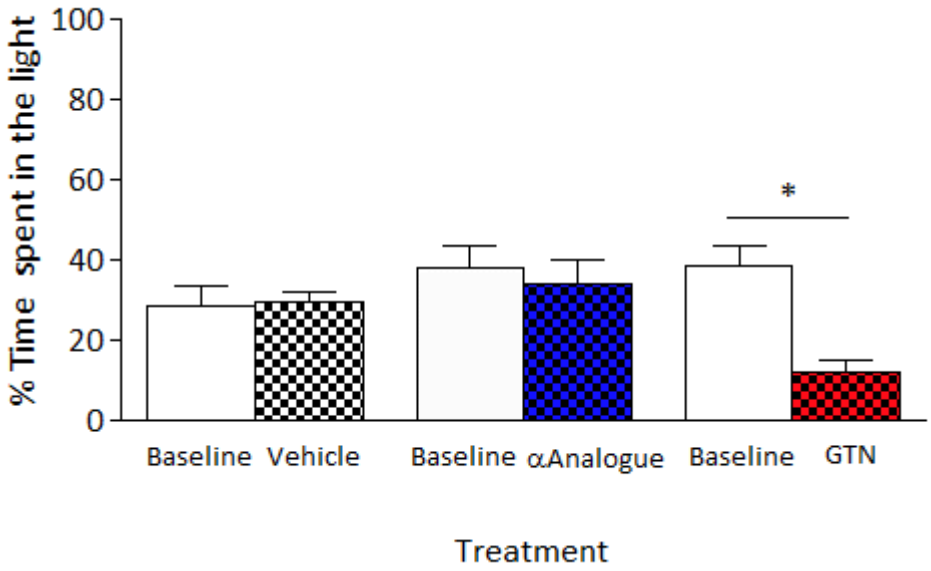
Supplemental Figure 3. Effect of daily systemic treatment with α-CGRP analogue (αAnalogue, 50nmol/kg) on cardiovascular haemodynamics in angiotensin II (AngII)-induced hypertension. Mice were infused with either AngII (1.1mg/kg/day) or control (saline) with osmotic pumps for 14 days and treated daily with vehicle or αAnalogue (50nmol/kg, *s.c.*). (**A)** Diastolic blood pressure, (**B)** mean blood pressure, (**C)** heart rate and (**D)** activity were measured by radiotelemetry. Results show measurement taken every 10 min, expressed as 6h average. Mice experience a 12/12h light/dark cycle, with the dark cycle shown in the grey striped area. Arrow represents the start of daily treatment. Results show mean ± SEM (n=4-7). *p<0.05, **p<0.01, ***p<0.001 vs vehicle-treated saline-infused mice; #p<0.05, ##p<0.01, ###p<0.001 for αAnalogue-treated mice AngII-infused vs vehicle-treated AngII-infused mice (Repeated measures 2-way ANOVA + Bonferroni *post hoc* test).



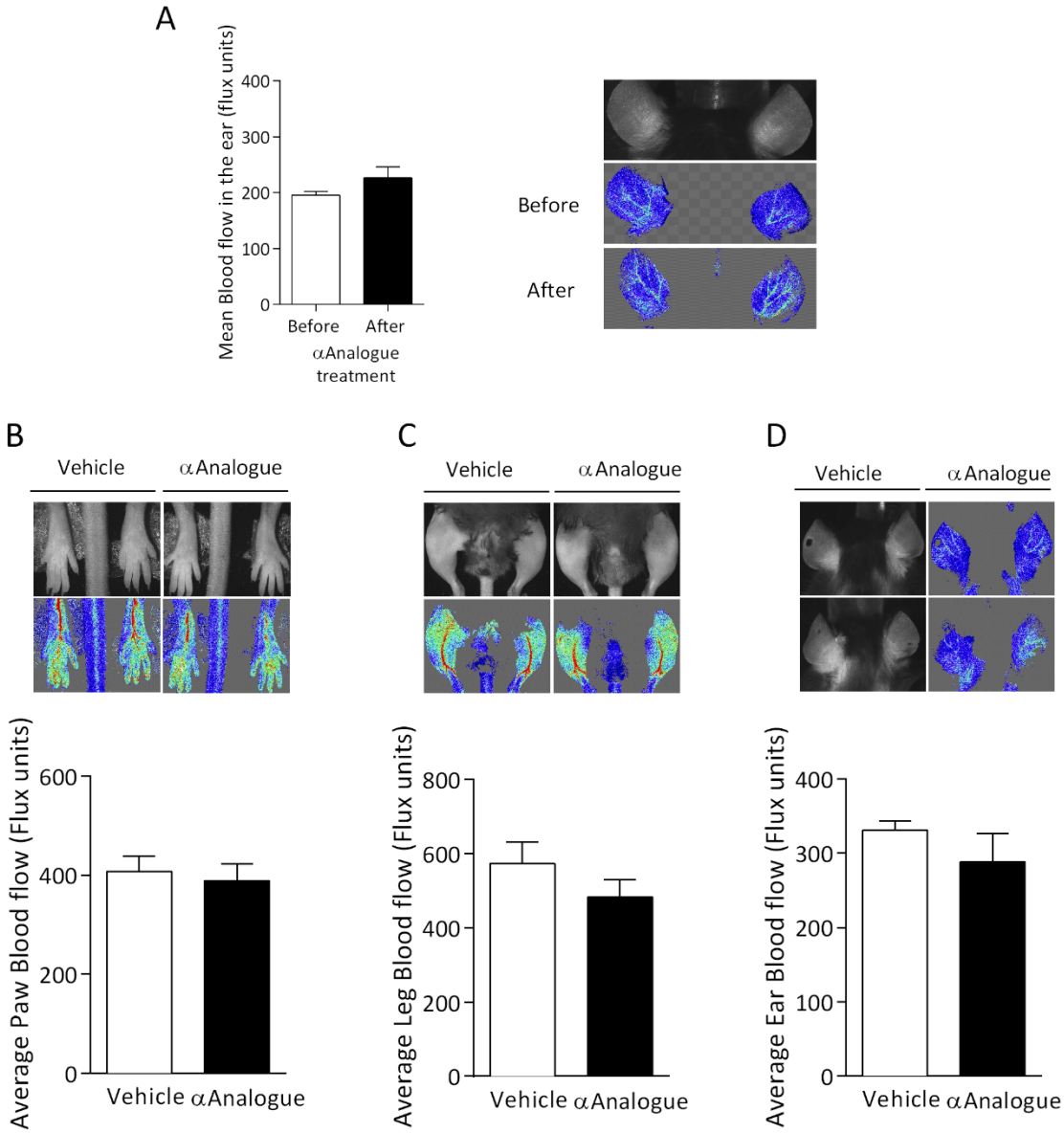
Supplemental Figure 4. Daily systemic treatment with the α-CGRP analogue (αAnalogue) produces a reproducible reduction in blood pressure in Angiotensin II (AngII)-induced hypertension in mice. 6h time course profile of systolic blood pressure following injection of vehicle or αAnalogue (50nmol/kg) at Day 1, 3, 7, 10 and 14 in mice infused with saline **(A and C)** or AngII **(B and D)**. **Left Panels,** Detailed analysis of SBP changes pre-and up to 6h post- αAnalogue or vehicle injection in mice infused with saline or AngII. **Right Panels,** Change (Δ) in SBP at Day 1 and Day 14 at 1h following injection from baseline (n=4-7). Results show mean ± SEM.



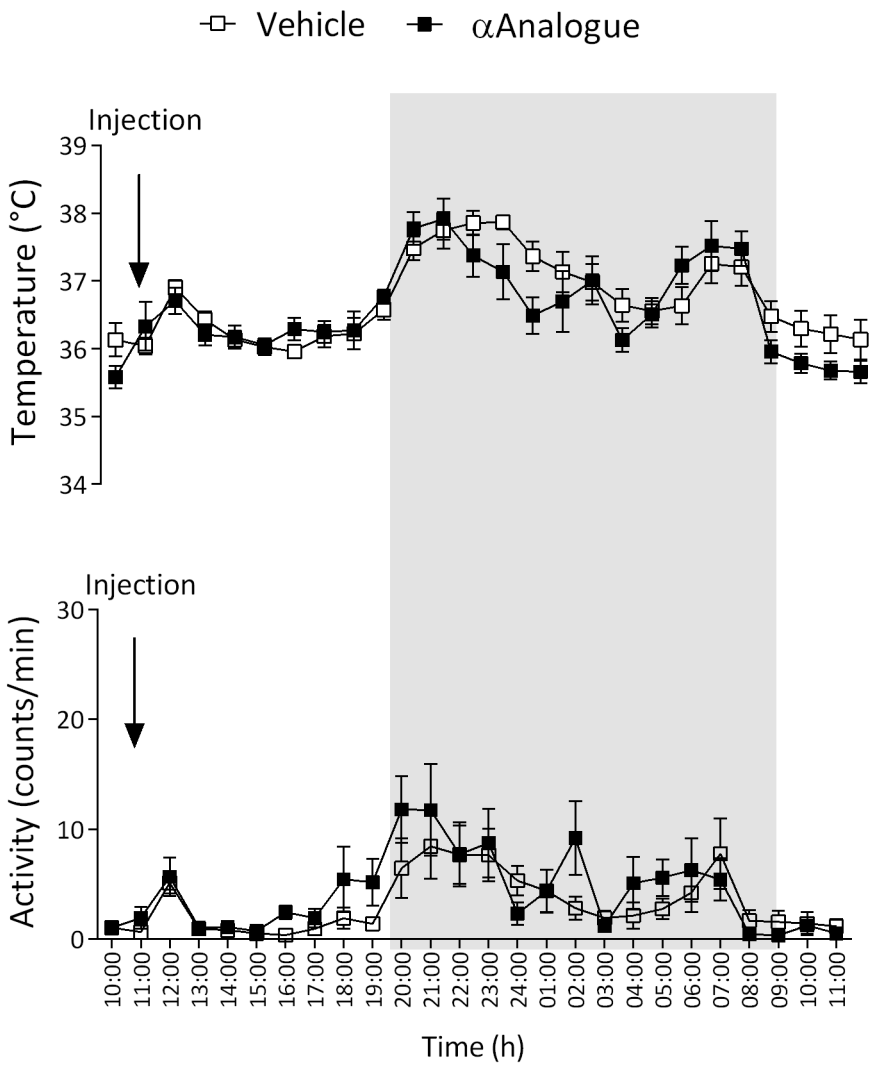
Supplemental Figure 5. Effects of the α-CGRP analogue (αAnalogue) or vehicle on (A-B) water consumption, (C) body weight and (D) food intake in Angiotensin II (AngII)-induced hypertension in mice. Baseline measurements of body weight, food and water intake were taken for 7 days prior to osmotic pump implantation. Mice were administered with osmotic pumps containing saline or AngII (1.1 mg/kg/day for 14 day). Measurements were taken daily. Data are mean ± S.E.M from n=5-7. AUC represents area under the curve results throughout the 14 day recording. Results show mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 versus vehicle-treated group, #p<0.05, ##p<0.01, ###p<0.001 versus αAnalogue-treated group (Repeated measures 2-way ANOVA + Bonferroni *post hoc* test for A,C-D and 2-way ANOVA + Bonferroni *post hoc* test for B).



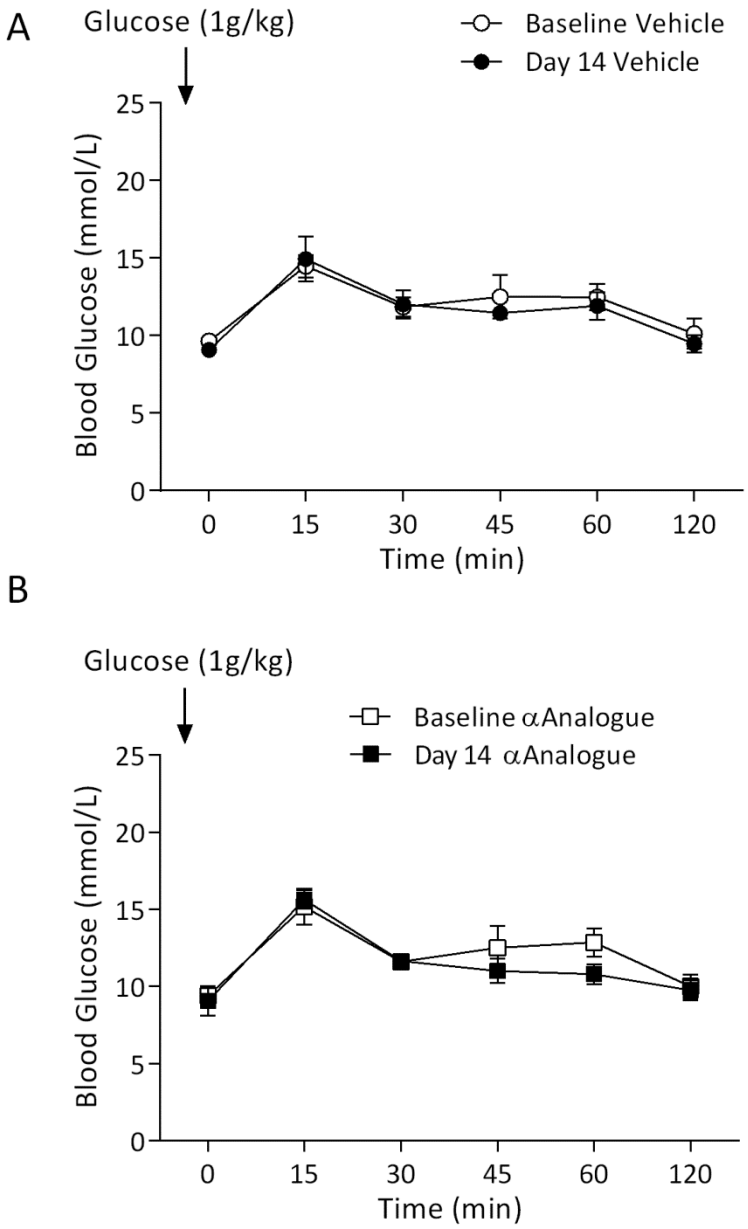
Supplemental Figure 6. Effects of the α-CGRP analogue (αAnalogue) on light-aversion. Mice were trained twice daily for 5 days to enable an even distribution to enter the dark covered zone or bright light uncovered area (n=6-7). On the test day, time (s) spent in the light (1000 lux) was recorded for 600s at baseline and 1h following administration of αAnalogue (50nmol/kg, *s.c.*) or glyceryl trinitrate (GTN, 352nmol/kg, *i.v.*). Results show % time spent in light in mice, mean ± SEM (n=6-7). *p<0.05 vs respective baseline (paired t-tests).



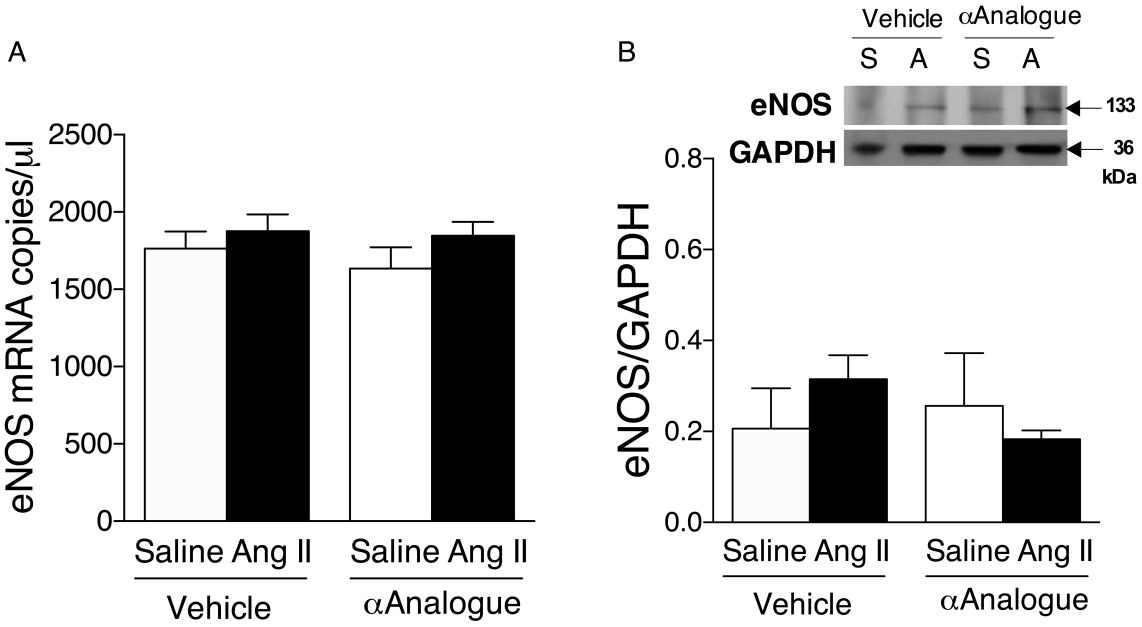
Supplemental Figure 7. Effects of the α-CGRP analogue (αAnalogue) on peripheral blood flow in mice. Mice were briefly anaesthetised with isoflurane (2%) and blood flow was monitored using Full-field Laser Perfusion Imager (FLPI) for 5 min. **(A)** Average blood flow in the ear vasculature at baseline (before) and 1h following a single treatment of αAnalogue (50nmol/kg, *s.c.*) in mice (n=6). Average blood flow in the **(B)** paw, **(C)** leg and **(D)** ear of mice at day 14 following daily treatment of vehicle or αAnalogue (50nmol/kg, *s.c.*). Representative FLPI pictures alongside grey/black 'photo' showing blood flow for vehicle and αAnalogue treatment. Results represent mean ± SEM for average measurement (flux units) for 5 min recording. p>0.05 ns (two-tailed Student's *t*-test).



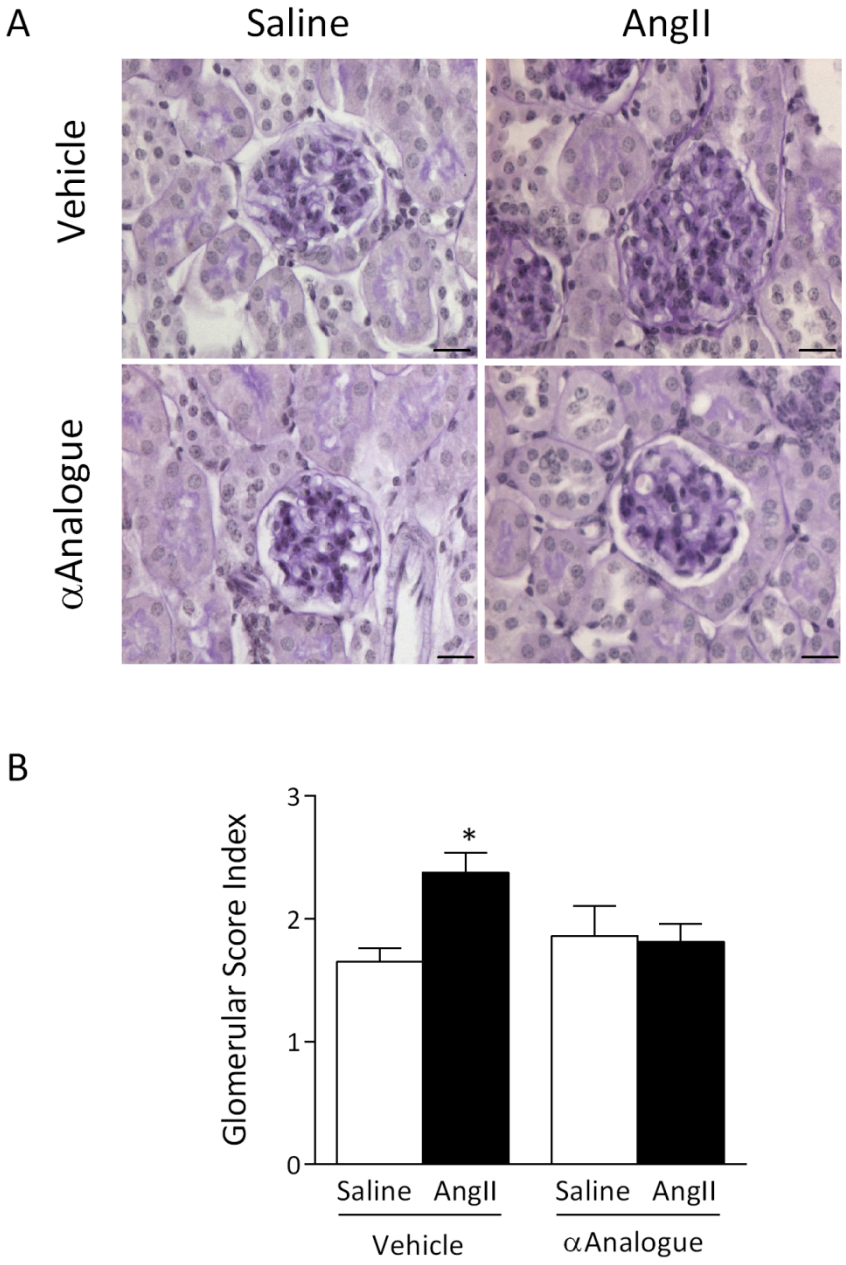
Supplemental Figure 8. Effects of α-CGRP analogue (αAnalogue) on core body temperature and activity in mice Core body temperature and activity recordings over 24h at baseline and following vehicle or αAnalogue (50nmol/kg, *s.c.*) treatment in conscious mice (n=5). Results represent average 1h recording, mean ± SEM. Arrow denotes time of treatment and grey area represents night/dark period. NS $p>0.05$ vs vehicle treatment (Repeated measures 2-way ANOVA + Bonferroni *post hoc* test).



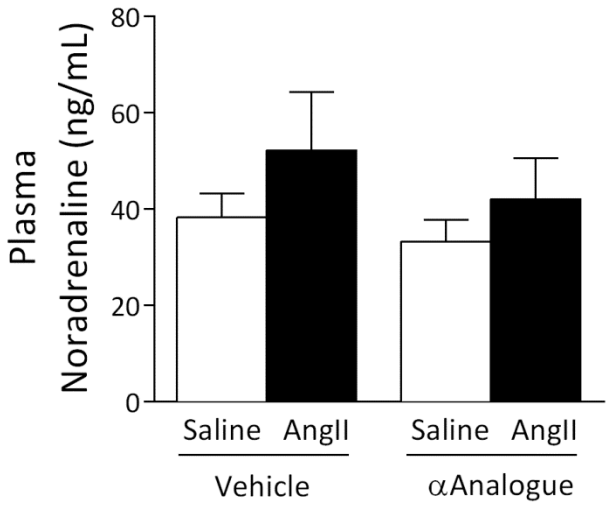
Supplemental Figure 9. Effects of cardiovascular active dose of α-CGRP analogue (αAnalogue) on glucose tolerance test in mice. Time course of plasma glucose concentrations (mmol/L) following systemic administration of glucose (1g/kg, *i.p.*) in mice treated daily with **(A)** vehicle (n=4) and **(B)** αAnalogue (50nmol/kg/day, *s.c.*, n=5) for 14 days. Results show mean ± SEM. Arrow denotes treatment time of glucose administration. NS $p>0.05$ vs baseline (Repeated measures 2-way ANOVA + Bonferroni post *hoc* test).



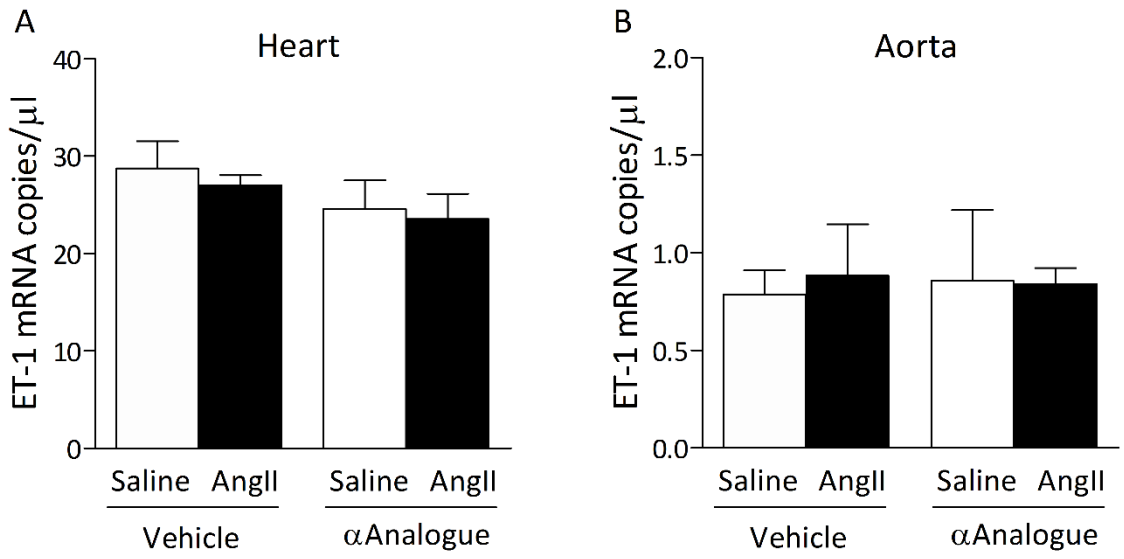
Supplemental Figure 10. Effects of α-CGRP analogue (αAnalogue) on vascular eNOS expression in mesenteric vessels. Mice were treated daily with the α-CGRP analogue (50nmol/kg, *s.c.*) or vehicle post AngII or saline infusion for 14 days. mRNA expression measured by qRT-PCR (n=4-6) and expressed as copy numbers per µl normalised to HPRT, B_2M and β-actin. Protein expression normalised by GAPDH and shown by immunoblotting (top panel) and densitometry analysis (bottom panel). (**A)** mRNA and **(B)** protein expression of endothelial nitric oxide synthase (eNOS). Results show mean ± SEM. ns $p>0.05$ (2-way ANOVA + Bonferroni *post hoc* test).



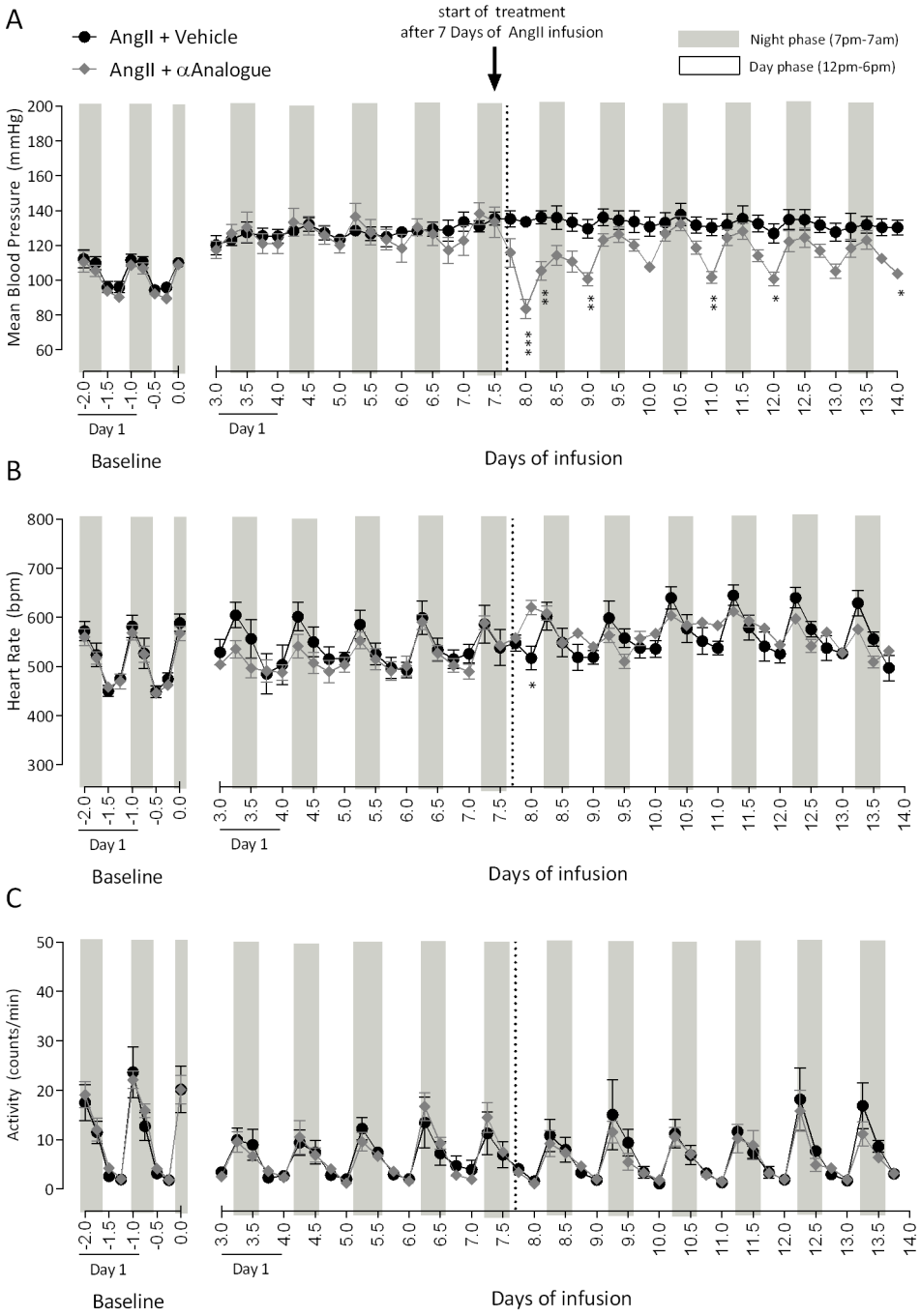
Supplemental Figure 11. Effects of α-CGRP analogue (αAnalogue) on morphological changes in the glomeruli in the kidney of Angiotensin II (AngII)-induced hypertension mice. Mice were treated daily with the αAnalogue (50nmol/kg, *s.c.*) or vehicle post AngII or saline infusion for 14 days. **(A)** Representative image showing glomerular mesangial pathology using PAS staining (20μm, scale bar). **(B)** Summarized glomerular matrix expansion by semi-quantification of scores in different groups. Results show mean ± SEM. *p<0.05 vs vehicle-treated (2-way ANOVA + Bonferroni *post hoc* test).



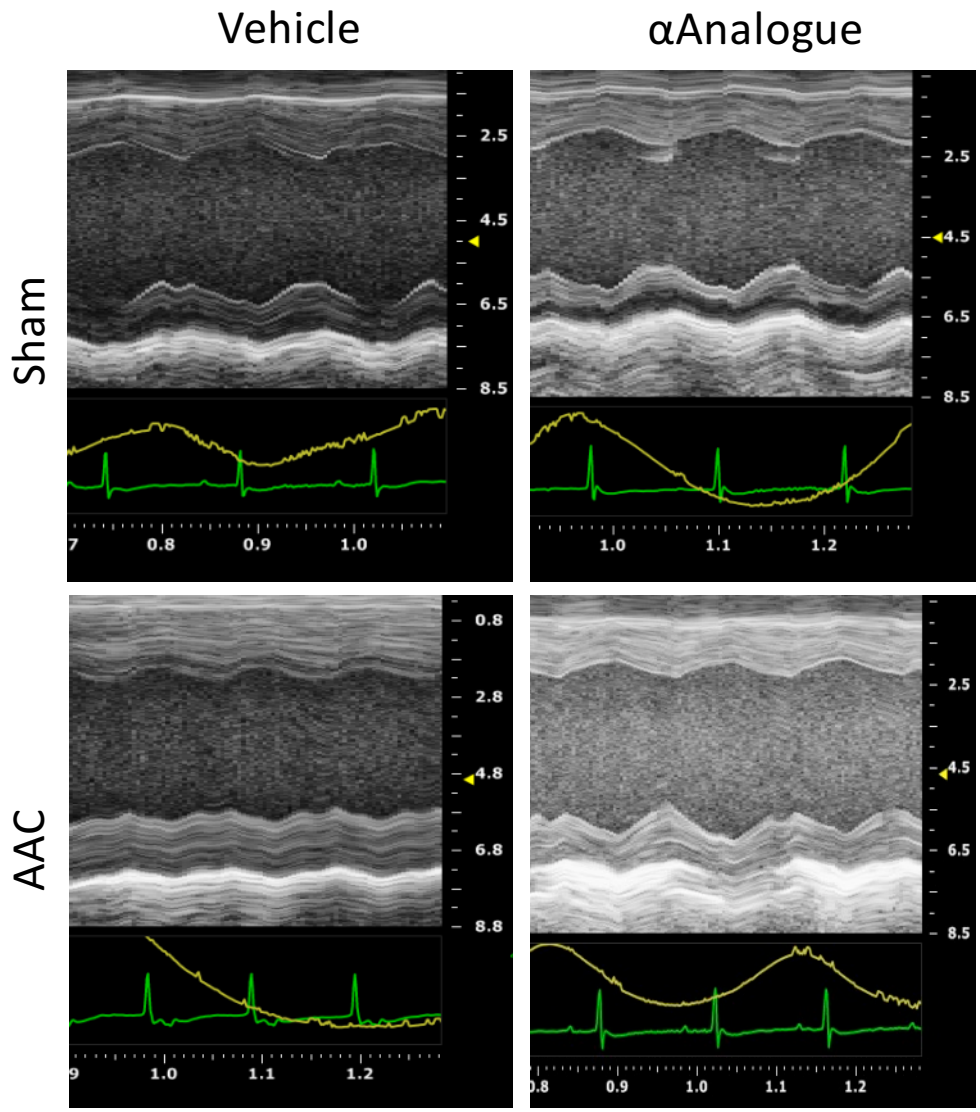
Supplemental Figure 12. Effects of α-CGRP analogue (αAnalogue) on plasma noradrenaline (NA) level in Angiotensin II(AngII)-induced hypertension mice. Mice were treated daily with the αAnalogue (50nmol/kg, *s.c.*) or vehicle post AngII or saline infusion for 14 days. NA was quantified using ELISA (n=6-9). Results show mean ± SEM. ns $p>0.05$ (2-way ANOVA + Bonferroni *post hoc* test).



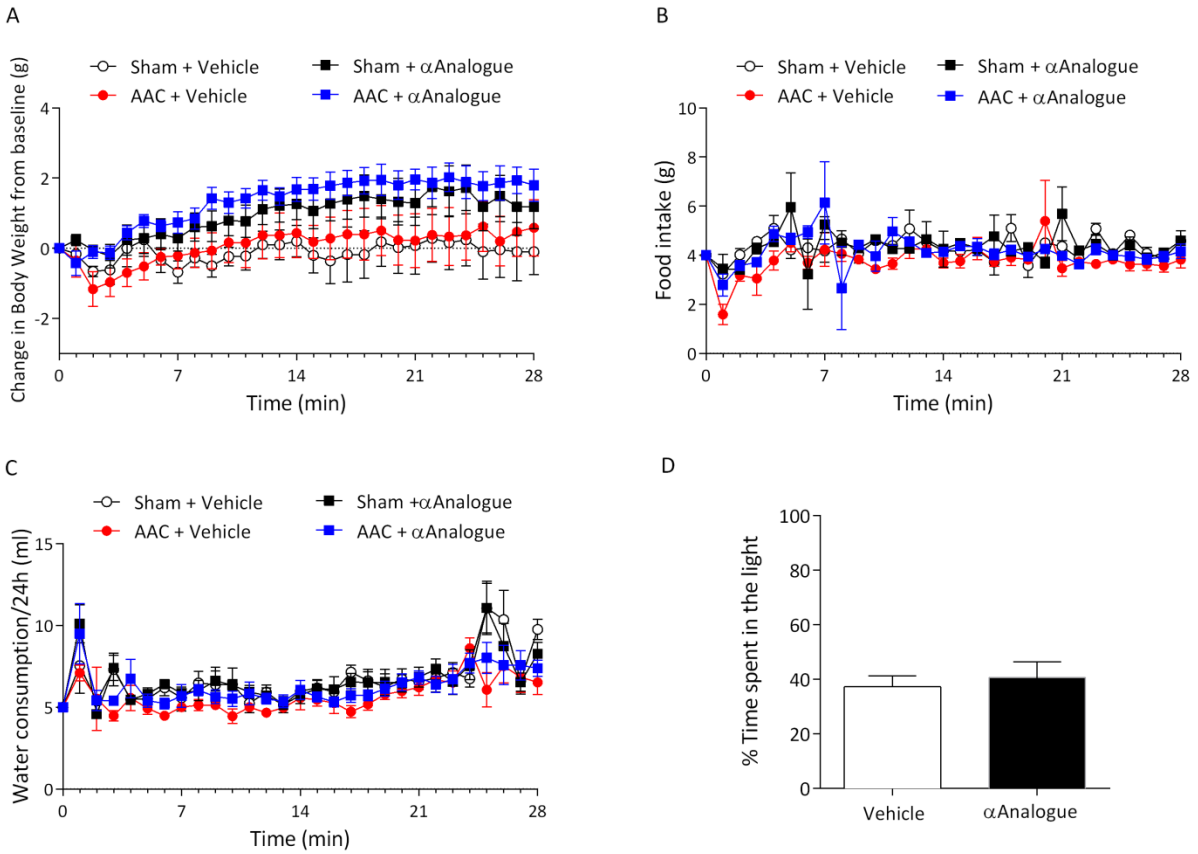
Supplemental Figure 13. Effects of α-CGRP analogue (αAnalogue) on mRNA expression of endothelial-1 (ET-1) level in Angiotensin II(AngII)-induced hypertension mice. Mice were treated daily with the αAnalogue (50nmol/kg, *s.c.*) or vehicle post AngII or saline infusion for 14 days. mRNA expression measured by qRT-PCR (n=6-7) for ET-1 in **(A)** heart and **(B)** aorta (n=4-11). Results expressed as copy numbers per µl normalised to HPRT, B2M and β-actin. Results show mean ± SEM. ns $p>0.05$ (2-way ANOVA + Bonferroni *post hoc* test).



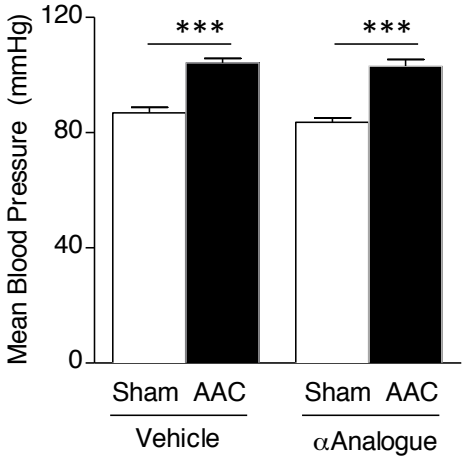
Supplemental Figure 14. Effect of daily treatment with α-CGRP analogue (αAnalogue, 50nmol/kg) on angiotensin II (AngII)-induced hypertensive mice. Mice were infused with AngII (1.1mg/kg/day) osmotic pumps for 14 days and treated with vehicle or αAnalogue (50nmol/kg) on Day 7-14 (n=4). **(A)** Mean blood pressure, **(B)** heart rate and **(C)** activity were measured by radiotelemetry. Results show measurement taken every 10 min, expressed as 6h average. Mice experience a 12/12h light/dark cycle, with the dark cycle shown in the grey striped area. Arrow indicates the start of daily treatment with vehicle or αAnalogue. *p<0.05, **p<0.01, ***p<0.001 vs vehicle-treated AngII-infused mice (Repeated measures 2-way ANOVA + Bonferroni *post hoc* test).



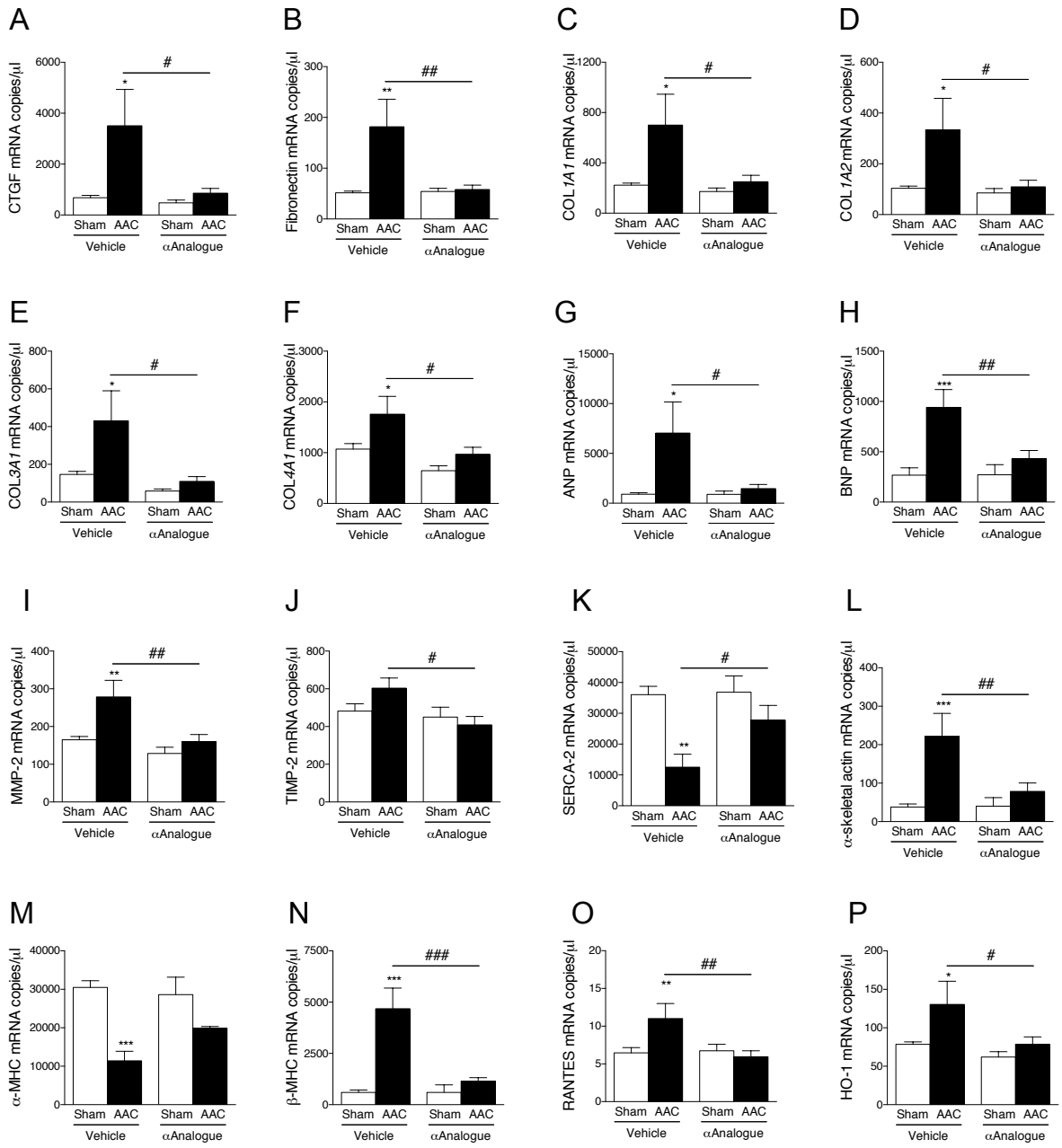
Supplemental Figure 15. Effect of daily treatment with α-CGRP analogue (αAnalogue) on abdominal aortic constriction (**AAC)-induced cardiac hypertrophy and heart failure in mice.** Mice were treated with vehicle or αAnalogue (50nmol/kg, *s.c.*) for 5 weeks post-surgery and cardiac function was assessed using echocardiography at week 5. Representative images of cardiac function and dimension using M-Mode echocardiography in the parasternal long axis view in mice after sham or AAC surgery with or without treatment.



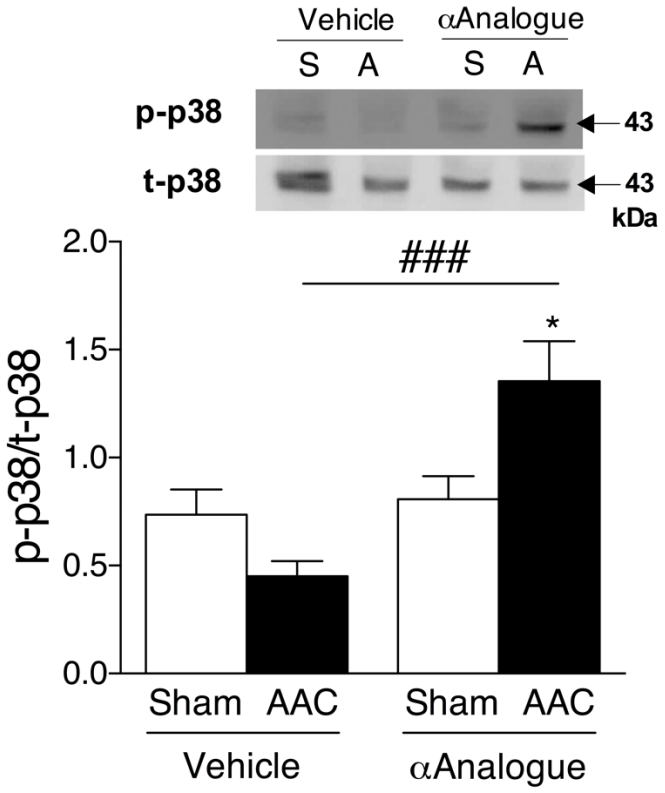
Supplemental Figure 16. Effects of the α-CGRP analogue (αAnalogue) or vehicle on (A) body weight, (B) food intake, (C) water consumption and (D) light aversion assay in abdominal aortic constriction-induced cardiac hypertrophy and heart failure. Mice were treated with vehicle or αAnalogue (50nmol/kg, *s.c.*) for 5 weeks post-surgery. Measurements were taken daily. Data are mean ± S.E.M from n=6-8. ns p>0.05 (Repeated measures 2-way ANOVA + Bonferroni *post hoc* test).



Supplemental Figure 17. Changes in blood pressure in vehicle or α-CGRP analogue (αAnalogue)-treated at 5 weeks following abdominal aorta constriction-induced cardiac hypertrophy and heart failure in mice. Blood pressure measurement obtained by carotid artery cannulation in isoflurane-anaesthetised mice treated daily with the αAnalogue (50nmol/kg, *s.c.*) or vehicle for 5 weeks (n=7-8). Results show mean $\pm$ SEM. ***p<0.001 vs vehicle treated sham mice (2-way ANOVA + Bonferroni *post hoc* test).



Supplemental Figure 18. Daily systemic treatment with α-CGRP analogue (αAnalogue) protects against abdominal aorta constriction (AAC)-induced cardiac fibrosis, hypertrophy, inflammation and oxidative stress. Following AAC, mice were treated daily for 5 weeks with vehicle or αAnalogue (50nmol/kg, *s.c.*). mRNA expression measured by qRT-PCR (n=6-7) for **(A)** CTGF, **(B)** fibronectin, **(C)** collagen type 1 α (COL*1A1*), **(D)** collagen type 1 α2 (COL*1A2*), **(E)** collagen type 3 α1 (COL*3A1*), **(F)** collagen type 4 α1 (COL*4A1*), **(G)** atrial natriuretic peptide (ANP), **(H)** brain natriuretic peptide (BNP), **(I)** matrix metalloproteinase-2 (MMP-2), **(J)** tissue inhibitor of metalloproteinase 2 (TIMP-2), **(K)** sarco-endoplasmic reticulum Ca^{2+} ATPase-2 (SERCA-2), **(L)** α-skeletal actin, **(M)** α-myosin heavy chain (α-MHC), **(N)** β-myosin heavy chain (β-MHC), **(O)** chemokine RANTES and **(P)** heme oxygenase-1 (HO-1) in heart (n=6-7). Results expressed as copy numbers per µl normalised to HPRT, B2M and β-actin. Results show mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs vehicle-treated sham mice; #p<0.05, ##p<0.01, ###p<0.001 vs vehicle-treated AAC mice (2-WAY ANOVA + Bonferroni *post hoc* test).



Supplemental Figure 19. Effects of α-CGRP analogue (αAnalogue) on the phosphorylation of p-38 mitogen-activated protein kinases (MAPK) in the heart of abdominal aortic constriction-induced cardiac hypertrophy and hear failure in mice. Mice were treated daily with the αAnalogue (50nmol/kg, *s.c.*) or vehicle post-surgery. Protein expression of phosphorylated p-38 was normalised to total p-38 (t-p38) and are shown by immunoblotting (top panel) and densitometry analysis (bottom panel) in heart tissues (n=6-8). Results show mean $\pm$ mean. *p<0.05 vs sham-treated, ###p<0.001 vs sham-treated (2-WAY ANOVA + Bonferroni *post hoc* test).

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