Protection of Angiotensin II–Induced Vascular Hypertrophy in Vascular Smooth Muscle–Targeted Receptor Activity-Modifying Protein 2 Transgenic Mice

Lihuan Liang, Christina W. Tam, Gabor Pozsgai, Richard Siow, Natalie Clark, Julie Keeble, Knut Husmann, Walter Born, Jan A. Fischer, Robin Poston, Ajay Shah, Susan D. Brain

Abstract—The vasodilator and vascular regulatory peptide adrenomedullin (AM), a member of the calcitonin gene-related peptide family of peptides, is predicted to play a pivotal protective role in cardiovascular dysfunction. The principle AM (AM1) receptor is composed of a G protein–linked calcitonin receptor-like receptor and a receptor activity-modifying protein (receptor activity-modifying protein 2). There is little knowledge of the receptors via which AM acts in diseases. Using smooth muscle-targeted receptor activity–modifying protein 2 transgenic mice with increased vascular density of functional AM1 receptors, we demonstrate that receptor activity-modifying protein 2 transgenic mice are not protected against angiotensin II–induced hypertension or cardiac hypertrophy. However, vascular hypertrophy, together with vascular cell adhesion molecule 1 and monocyte chemotactic protein 1 expression, is significantly reduced in the aortic walls of transgenic mice, as determined by histological techniques. This indicates that the AM1 vascular smooth muscle receptor can mediate local protection in vivo. This is supported by proliferation studies in cultured smooth muscle cells. By comparison, levels of hypotension and inflammation in a shock model were similar to those in wild-type mice. Thus, a role of the AM1 receptor in the vasoactive component could not be detected, and evidence is provided to show that the hypotensive response to AM is subject to desensitization in vivo. The finding that the vascular smooth muscle AM1 receptor acts at a local level to protect against hypertension-induced vascular hypertrophy and inflammation provides evidence that targeting this receptor may be a beneficial therapeutic approach. (Hypertension. 2009;54:1254-1261.)

Key Words: RAMP2 ■ adrenomedullin ■ hypertension ■ vascular hypertrophy ■ transgenic mice ■ vascular smooth muscle

Adrenomedullin (AM) is a member of the calcitonin gene-related peptide (CGRP) family of peptides that includes intermedin and amylin.1 AM and CGRP possess potent vasodilator activity, and AM is ~10 to 100 times less potent than CGRP. However, there is also strong evidence showing that AM possesses vascular development and remodeling properties.2–6 It is considered to play a pivotal role in cardiovascular regulation. AM expression is upregulated in vascular tissues in response to inflammatory cytokines7 and hypoxia.8 Moreover, plasma levels are raised in cardiovascular conditions that include heart failure and sepsis.9,10 Studies in AM transgenics and heterozygous knockouts have revealed a protective function of this peptide,1 as shown in sepsis,4,11 and collateral vessel development in ischemia.12 Furthermore, beneficial effects of exogenous AM were detected in ischemia, hypovolemic shock, and endotoxemia.13–16 Thus, upregulation of the peptide correlates with important cardiovascular activities, although little is known about the receptors involved in these responses.

Receptors for AM and CGRP constitute a unique family of G protein–linked receptors. They consist of the calcitonin receptor-like receptor (CL) with 7 transmembrane domains and 1 of 3 associated receptor-activity-modifying proteins (RAMPs). The heterodimerization of the CL with RAMP1, RAMP2, or RAMP3 leads to the formation of a CGRP, AM1, or AM2 receptor, respectively.17 Studies in a range of species provide evidence that AM is a vasodilator agonist at both AM and CGRP receptors but that CGRP primarily acts via the CL/RAMP1 CGRP receptor. However, differential receptor activities and signaling occur in different vascular cells and beds.3 As a consequence, little is known regarding the cardiovascular relevance of the specific AM receptors in vivo. Moreover, few studies to date have been able to use tools that enabled specific associations between RAMP in

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specific cells or tissues and peptide physiology/pathology to be elucidated.

We have developed and investigated RAMP2 transgenic (RAMP2 TG) mice with upregulation of RAMP2 predominantly in smooth muscle-containing tissues.18 They exhibited an enhanced hypotensive response to AM compared with wild-type (WT) mice, although a normal baseline blood pressure and a similar response to CGRP were observed in both populations of mice.18 This implies that RAMP2 overexpression is associated with an increased density of functionally relevant AM1 (CL/RAMP2) receptors.18 We now demonstrate that RAMP2 TG mice, unlike WT littermates, are protected against vascular hypertrophy and vascular inflammation caused by chronic angiotensin II (ATII) infusion over 13 days, although hypertension is similar in both WT and RAMP2 TG mice. Furthermore, the AM1 receptor was not found to mediate the hypertensive response to ATII in either an angiotensin-induced hypertensive model or a lipopolysaccharide (LPS)-induced hypotensive model.

### Methods

Please see the online Data Supplement for the expanded methods section at http://hyper.ahajournals.org.

### Generation of RAMP2 TG Mice

A SigMyc-mRAMP2 cDNA construct with an α-actin promoter was introduced into the genome of C57BL/6 mice.18 Heterozygous RAMP2 TG offspring and WT littermates were used, which were obtained by mating WT and heterozygous TG. The genotype of the mice was determined by PCR.18

### ATII-Induced Hypertension

Osmotic minipumps containing ATII (infusion rate: 0.9 mg/kg per day) or vehicle were implanted in the midscapular region of WT and RAMP2 TG mice (25 to 35 g, 12 to 16 weeks old, matched for age and sex). Blood pressure was measured using tail-cuff plethysmography. Animals were humanely killed by anesthetic overdose with cervical dislocation, and cardiac hypertrophy was assessed. Weights were expressed as ratios of the body weight.

### Assessment of Vascular Hypertrophy

For histological analysis, tissue sections were stained with either hematoxylin and eosin or Masson’s trichrome. For monocyte chemotactic protein 1 (MCP-1) detection, paraffin aortic sections were stained with either hematoxylin and eosin–stained cross-sections revealed a 2.0-fold increase in collagen deposition, indicated no significant difference in fibrosis thickness, but differences were observed in medial thickness between the ATII-treated WT and TG animals (Figure 2D and 2E). VCAM-1 and MCP-1 immunostaining, indicating vascular inflammation, was observed in the vascular layer of ATII-treated WT mice. Only low immunoreactivity could be detected in aortic walls of RAMP2 TG animals (Figure 3).

### Vascular Smooth Muscle Cell Culture

Primary cultures of vascular smooth muscle cells (VSMCs) were obtained from aortic explants of WT and RAMP2 TG mice and cultured to a third passage.19 Then VSMCs were trypsinized and placed into 24-well plates at a density of 10^5 cells per well. The cells were quiesced for 24 hours before the addition of drugs. Cell proliferation, determined by cell number, was compared in the presence or absence of ATII (10^{-7} mol/L) ± AM22-52 (10^{-7} mol/L) for 24, 48, and 72 hours.

### LPS-Induced Hypotension and Hypothermia

LPS (serotype 0127:B8), 2.25×10^6 units/mg in saline (10 mL/kg), was injected IP into WT and RAMP2 TG animals. Blood pressure and body temperature were monitored just before and 0.5, 1.5, and 4.0 hours after LPS administration. The NO_2^-/NO_3^- content of peritoneal exudate lavage fluid was determined (Griess assay) for NO levels.20 Calcium-independent inducible NO synthase (iNOS) activity was measured in lung tissue extracts.20

### Measurement of Blood Pressure in Anesthetized Mice

WT and RAMP2 TG mice (25 to 35 g, matched age and sex) were anesthetized, and a tracheotomy was performed. They received either human α-CGRP in 0.01% BSA or human AM1,52 given alone or with AM22-52 (IV; see the online Data Supplement).

### Data Analysis and Statistics

Data are shown as mean±SEM. Statistics was performed by ANOVA followed by Bonferroni or Tukey post hoc test. Some studies involving exogenous peptides were analyzed using an unpaired or paired t test. P<0.05 was considered statistically significant.

### Results

#### Effect of ATII on Blood Pressure and Cardiac Hypertrophy in WT and RAMP2 TG Mice

Basal blood pressure did not differ between WT (117.8±3.2 mm Hg) and RAMP2 TG mice (119.7±4.2 mm Hg, mean±SEM; n=8 to 16), as reported previously.18 However, ATII (0.9 mg/kg per day for 13 days) increased blood pressure over 13 days in a similar manner in both WT and RAMP2 TG mice (Figure 1A). ATII also induced significant and similar left ventricular hypertrophy in both genotypes (Figure 1B and 1C). The results were analyzed for sex, but no differences were observed, so results were pooled.

#### Effect of ATII on Vascular Hypertrophy in WT and RAMP2 TG Mice

ATII caused hypertrophy of the aorta in WT mice, which was not observed in RAMP2 TG mice. Analysis of hematoxylin and eosin–stained cross-sections revealed a 2.0-fold (P<0.01) increase in area and a 1.5-fold (P<0.01) increase in thickness of the aortic wall of ATII-treated WT as compared with RAMP2 TG mice (Figure 2A through 2C). Masson’s trichrome staining, differentiating between smooth muscle and collagen deposition, indicated no significant difference in fibrosis thickness, but differences were observed in medial thickness between the ATII-treated WT and TG animals (Figure 2D and 2E). VCAM-1 and MCP-1 immunostaining, indicating vascular inflammation, was observed in the vascular layer of ATII-treated WT mice. Only low immunoreactivity could be detected in aortic walls of RAMP2 TG animals (Figure 3).

#### Culture of WT and RAMP2TG Cells

To determine the effect of RAMP2 overexpression on cell proliferation, studies were carried out in cultured VSMCs. Experiments revealed that the RAMP2 TG cells grew signif-
significantly slower than WT cells (Figure 4A). After stimulation with ATII (10⁻⁷ mol/L), the cell number was significantly increased in WT cells (Figure 4B) as early as 24 hours but not in TG cells (Figure 4C). The possible involvement of endogenous AM in VSMC growth was investigated using the AM1 receptor antagonist AM22-52 (10⁻⁴ mol/L). The antagonist was found to significantly enhance the growth of TG cells in the presence of ATII, as shown in Figure 4C and 4D. This is not observed with WT cells (Figure 4B). Results are also depicted as a ratio of proliferation induced by ATII+AT22-52 alone, compared with ATII alone (Figure 4D).

LPS-Induced Hypotension in WT and RAMP2 TG Animals

The possibility that RAMP2 was important in hypotensive responses rather than hypertensive responses was investigated using an LPS-model of endotoxic shock. LPS (IP) induced a profound hypotensive response in both WT and RAMP2 TG mice (Figure 5A) and a decrease in body temperature (Figure 5B). The levels of the inflammatory marker NO, measured as nitrite, were raised in response to LPS, with similar levels observed in WT and RAMP2 TG tissues (Figure 5C). The activity of the calcium-independent iNOS was similar in lung tissue extracts of WT and RAPM2 TG mice, as determined in the presence of EGTA using the l-citrulline assay. It was blocked by the NO synthase inhibitors N⁶-monoo-methyl-L-arginine and Nω-nitro-L-arginine methyl ester (Figure 5D). These results indicate that the vascular smooth muscle RAMP2 and, thus, the AM1 receptor have the potential to play a significant role in protecting against endotoxemia. Furthermore, studies of VSMCs in culture in the presence of ATII revealed reduced proliferation of RAMP2 TG cells compared with WT cells. In addition, the AM1 receptor antagonist AM22-52 enhanced RAMP2 TG VSMC proliferation. This suggests that endogenous smooth muscle–derived AMs and the AM1 receptor have the potential to play a significant role in protecting against the onset of vascular remodeling in response to ATII-evoked chronic hypertension. By comparison, the RAMP2 overexpression did not influence either the raised systemic blood pressure observed in ATII-mediated hypertension or the resulting cardiac hypertrophy. A lack of effect of the AM1 receptor on pathological hemodynamic responses was additionally observed in an LPS-induced model of sepsis.

Desensitizing Effect of AM on Hypotensive Responses in C57BL/6 Mice

The lack of difference in WT and RAMP2 TG mice on the blood pressure changes in the models described above led us to investigate the effect of AM as compared with CGRP on blood pressure changes. Evidence for a selective desensitization of AM vasoactive receptors was found (see the online Data Supplement). Both AM and CGRP produced dose-dependent hypotension, but AM exhibited a selective desensitization, such that repeated applications could not be given to the same mouse (see Figure S1 in the online Data Supplement). The AM receptor antagonist AM22-52 (30 nmol/25 g, a dose chosen from preliminary experiments) significantly inhibited AM-induced hypotension when given as a coinjection (−29.2±4.8% AM alone compared with −16.3±1.8% AM+AM22-52; P<0.05; n=4 per group; data not shown). Figure S2 shows that mice exhibited desensitization when a second dose of AM was given 1 hour later. This was not observed with a similar hypertensive dose of CGRP or in experiments to evaluate whether AM desensitized to CGRP or vice versa. RAMP2 WT mice showed significant desensitization to a second application of AM, but desensitization was not observed in RAMP2 TG mice (see Figure S3A and S3B). Experiments with CGRP did not provide evidence of desensitization in these mice (see Figure S3C and S3D).

Discussion

We have used transgenic mice with targeted vascular smooth muscle overexpression of RAMP2, shown previously to correlate with raised levels of a functional AM1 receptor (CL/RAMP2). The major finding is that this receptor has a key vascular protective role in vivo in that the RAMP2 TG mice were protected against ATII-mediated aortic vascular hypertrophy and inflammation. Furthermore, studies of VSMCs in culture in the presence of ATII revealed reduced proliferation of RAMP2 TG cells compared with WT cells. In addition, the AM1 receptor antagonist AM22-52 enhanced RAMP2 TG VSMC proliferation. This suggests that endogenous smooth muscle–derived AMs and the AM1 receptor have the potential to play a significant role in protecting against the onset of vascular remodeling in response to ATII-evoked chronic hypertension. By comparison, the RAMP2 overexpression did not influence either the raised systemic blood pressure observed in ATII-mediated hypertension or the resulting cardiac hypertrophy. A lack of effect of the AM1 receptor on pathological hemodynamic responses was additionally observed in an LPS-induced model of sepsis. AM1 receptor desensitization, involving vasoactive receptors
in vivo, was observed, but whether this links to blood pressure changes is unknown.

These findings provide, for the first time, a key insight into the importance of the vascular smooth muscle AM1 receptor in vivo. Circulating AM levels are raised in a range of cardiovascular diseases, and AM is known to be produced from multiple cardiovascular cell types. The mRNA for RAMP2 is upregulated in models of cardiovascular disease. The results of the present study complement previous evidence of a protective role of the AM peptide. Heterozygous AM gene knockout mice with lower endogenous AM levels than normal animals exhibit increased damage in models of cardiovascular disease compared with the respective controls. Recently, AM transgenic mice have been shown to be protected against vascular hypertrophy without modulation of blood pressure in ATII-induced hypertension. Indeed, an elevated plasma AM level has been suggested to be a predictor of future cardiovascular events in high-risk patients. However, it should be noted that the levels of the AM-related peptide intermedin, which is an agonist at the CGRP family of receptors (including the AM1 receptor), is also raised in a rat model of pressure overload. Thus, AM1 receptor activity may be influenced by >1 agonist.

The histological analysis of the mouse aorta demonstrated a clear decrease in the width of the VSMC layer of ATII
RAMP2 TG mice compared with WT mice. Furthermore, an anti-inflammatory effect of the AM1 receptor is indicated from immunohistochemical studies, because the adhesion molecule VCAM-1 and mediator MCP-1 were upregulated in the WT ATII-treated mouse aorta compared with that from RAMP2 TG mice. Thus, our results indicate that the important function of AM via the AM1 receptor is found by reducing smooth muscle proliferation and the development of an inflammatory vascular phenotype as hypertension develops.

Several mechanisms for the protective effect of AM in cardiovascular disease have been put forward. AM has been shown to protect against ATII-induced migration and proliferation of VSMCs through mechanisms involving inhibition of free radical formation in culture, although some data have been conflicting with positive,26,27 as well as negative,28 effects reported. Fukai et al29 have presented evidence that either the AM1 or the AM2 receptor rather than the CGRP receptor, is involved. AM was shown to inhibit ATII-induced proliferation of human aortic smooth muscle cells via a cAMP/protein kinase A–dependent mechanism,30 but the role of AM in the VSMC proliferation response was complex and depended on the state of the cells.30 AM22-52 inhibited proliferation induced by quiescent cells but enhanced growth stimulated by ATII in the presence of AM.30 Here, we demonstrated that the RAMP2 TG VSMCs grew more slowly than WT cells, whether in the presence or absence of ATII, under culture conditions (5% FCS) used to compare growth of the WT and TG cells. AM22-52 was not tested on this, but AM22-52 was able to significantly enhance ATII-stimulated proliferation in RAMP2 TG cells compared with WT cells. These results suggest that endogenous AM is able to inhibit VSMC growth and that this is likely to be mediated via the increased levels of the functional AM1 receptor.

Having discovered that the hypertension was similar in WT and RAMP2 TG animals, we investigated the role of this receptor in LPS-induced hypotension for 2 reasons. First, sepsis has been shown to produce some of the highest levels of AM, as determined in a range of rodent and human studies,1,31 and AM is protective in sepsis.4,11 Second, the deterioration in sepsis is associated with hypotension and inflammation.32 Sepsis was similar in WT and RAMP2 transgenics in all of the parameters investigated. Shindo et al4 investigated AM transgenic mice and concluded that AM plays a protective role in sepsis. The combined results indicate a role for a non-VSMC AM1 receptor, such as the endothelial AM1 receptor, in protecting against sepsis.

AM shares with other peptides the ability to influence vascular tone while also exhibiting vascular protective mechanisms.33 Naïve RAMP2 TG mice demonstrate an enhanced hypotension to bolus AM.18 There is little knowledge of the relationship between RAMP activity and AM-mediated

Figure 3. Effect of ATII (0.9 mg/kg per day for 13 days) on VCAM-1 and MCP-1 expression in the aortic wall in WT and RAMP2 TG mice. A, Representative immunohistochemical staining of VCAM-1 in the thoracic aorta wall at ×20 and ×650, and negative control (c). B, Representative staining of MCP-1 in aorta wall at ×100 and ×400, and negative control (c). C, Percentage area of VCAM-1 (n=3 to 6) and (D) MCP-1 (n=3) expression to total area of the aorta. Data are presented as mean±SEM. ***P<0.001 compared with WT vehicle-treated mice and ###P<0.001 compared with WT ATII-treated mice.
pathological responses. Here, acute IV hypotensive doses of AM led to a tachyphylactic-like response that was not observed with IV CGRP, was inhibited by AM22-52, and did not show heterologous desensitization with CGRP. This tachyphylactic-like response has not been demonstrated previously in vivo. The desensitization of the AM1 receptor may play a role in the lack of vasoactive changes observed in the ATII model or simply may not be involved in the blood pressure regulation. On the other hand, RAMP2 WT mice showed a selective desensitization to AM, whereas the

Figure 4. Effect of ATII with or without AM22-52 compared with PBS on the growth of VSMCs cultured from WT and TG mice. Cell numbers were determined at 24, 48, and 72 hours. A, Growth of VSMCs in DMEM supplemented with 5% FCS; B, WT VSMCs were treated with PBS or ATII (10⁻⁷ mol/L) ± AM22-52 (10⁻⁷ mol/L); C, TG VSMCs were treated with PBS or ATII (10⁻⁷ mol/L) ± AM22-52 (10⁻⁷ mol/L); D, Ratio of proliferation induced by ATII + AM22-52 compared with ATII. Each point presents the mean of 3 separate experiments run in triplicate on 3 separate cultures. Data are presented as mean ± SEM. *P<0.05 and ***P<0.001 compared with other treatment group(s) at the same time point. ##P<0.01 and ###P<0.001 compared with vehicle group.

Figure 5. Effect of LPS (5 mg/kg; IP) on blood pressure, rectal temperature, nitrite levels, and lung nitric oxide synthase (NOS) activity in WT and RAMP2 TG mice. A, Percentage of change in mean arterial blood pressure (MAP) from baseline. B, Rectal temperature assessed as percentage of change from baseline. C, NO levels were assessed as total nitrate/nitrite in peritoneal exudate. D, iNOS activity in lung tissue extracts measured as L-citrulline production in the absence or presence of the iNOS inhibitors N₆-nitro-L-arginine methyl ester (L-NAME)/NG-mono-methyl-L-arginine (L-NMMA) mixture (300 μmol/L each) or EGTA (0.5 μmol/L). Data are expressed as mean ± SEM. *P<0.05, **P<0.01, or ***P<0.001 compared with vehicle-treated control animals. ^P<0.05 compared with INOS activity in the absence of inhibitors in lung tissue extracts of LPS-treated mice; n=4 to 9.
RAMP2 TG mice did not. The larger reserve of functional AM1 receptors appears to have protected against the loss of the hypotensive response in these TG mice. However, it is noted that circulating levels of AM in disease are substantially less than those needed to induce a hypotensive response. Therefore, these results, although of interest, may be independent of blood pressure changes in hypertension.

In conclusion, studies of the functional consequences of the AM1 receptor (composed of CL and RAMP2) in pathophysiological studies in vivo have been hindered by a lack of selective blocking regimes and the understanding that CL and/or RAMP homozygous knockouts are fatal phenotypes. Our approach, using RAMP2 TG mice, provides novel and important evidence that the vascular smooth muscle AM1 receptor plays a key role in protecting against vascular hypertrophy and inflammation. The results suggest that it may be an important target for developing novel therapeutic approaches. On the other hand, the vascular AM1 receptor does not contribute directly to blood pressure regulation in these diseased models.

Perspectives

There is evidence from a range of sources that AM may play a pivotal role in disease. However, few studies have been able to associate the expression of RAMP receptor components in specific cells or tissues with either physiological or pathological mechanisms. Here, using unique RAMP2 transgenic mice that overexpress RAMP2 predominantly in vascular smooth muscle, evidence is given that the vascular smooth muscle AM1 receptor may be important for vascular protection against damage that occurs during cardiovascular diseases, while not influencing blood pressure changes. This suggests that tissue-specific targeting of this receptor via either genetic or smooth muscle–specific means may provide novel therapies.

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Disclosures

None.

References


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PROTECTION OF ANGIOTENSIN-II INDUCED VASCULAR HYPERTROPHY IN VASCULAR SMOOTH MUSCLE- TARGETED RAMP2 TRANSGENIC MICE

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Running Title: Responses to AM in RAMP2 transgenic mice

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Supplement Materials and Methods

Animals
Experiments were performed under the UK Animals (Scientific Procedures) Act, 1986. Mice were maintained on normal diet, with free access to food and water, in a controlled environment. Either wild type (WT) and RAMP2 TG mice were used generated as below or female CD1 mice (25-30g) were purchased from Charles River Ltd., UK. All agents were from Sigma-Aldrich UK, unless stated.

Generation of RAMP2 TG mice
A SigMyc-mRAMP2 cDNA construct with an alpha-actin promoter was introduced into the genome of C57BL/6 mice (WT). Heterozygous RAMP2 TG offsprings which were used in the following experiments were obtained by mating WT and TG. The genotype of the mice was determined by polymerase chain reaction (PCR).

Angiotensin II-induced hypertension and assessment of cardiac hypertrophy
Osmotic minipumps (Model 1002, Alzet, USA) containing angiotensin II (ATII; infusion rate 0.9 mg/kg/day) or vehicle (0.01% 10 mmol/l acetic acid in saline) were implanted in the mid-scapular region of WT and RAMP2 TG mice (25-35 g, 12 – 16 weeks old, matched for age and sex). Buprenorphine hydrochloride (Reckitt Benckiser, UK, 50 \( \mu g/kg \)) was administered i.p. for post-operative pain relief. Blood pressure was monitored using tail cuff plethysmography (Coda6 System, Kent Scientific, USA) in conscious mice in a warmed (25 ºC) room, prior and at 3, 6, 9 and 13 days following implantation. Animals were humanely killed by anaesthetic overdose with cervical dislocation and cardiac hypertrophy was assessed by weighing the heart and also the isolated ventricles. Weights were expressed as ratios of the body weight.

Assessment of vascular hypertrophy
For histological analysis, the thoracic aorta were dissected, placed in 10% formalin and embedded in paraffin. Tissue sections (4 \( \mu m \)) were stained with either haematoxylin and eosin (H&E) or Masson’s Trichrome. Aortic wall width, fibrosis thickness, aortic medial thickness (mean of 8 measurements taken from at least 2 sections for each of 3 mice/group) and area (mean of at least 5 sections for each of 3 mice/group), were measured by Image-pro plus software. Mesenteric microvessels were also examined by H&E staining for evidence of vascular hypertrophy, but no change in either arteriolar diameter, or wall width was found.

Immunohistochemistry
The presence of vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemotactic protein-1 (MCP-1) was examined by immunohistochemistry in thoracic aorta. For VCAM-1 staining, fresh isolated aortic samples were immersed in OCT compound and stored at -70 ºC until further analysis. Serial fresh frozen aortic cross sections (10 \( \mu m \)) were stained with rabbit anti-human VCAM-1 antibody (1:50 dilution; sc-8304, Santa Cruz Biotechnology, USA) and visualised with Envision plus System-HRP (DAB), from Dako Cytomation, Denmark. For MCP-1 expression, paraffin aortic sections (5\( \mu m \)) were immunostained with goat anti-MCP-1 antibody (1:50, sc-1785, Santa Cruz Biotechnology, USA). Then the MCP-1 staining was amplified by using the VECTASTAIN Elite ABC kit (pk-6105, Vector Laboratories). The percentage of the overall area of brown stained sites representing VCAM-1 or MCP-1 compared to the total area of the field of vision was quantified. The mean of area percentages of 3 sites was calculated for each section. Two-three sections were used from each of 3-6 mice in a group. All the histological measurements were performed by Image Pro Plus software or Cell^P Olympus software.
Vascular smooth muscle cell culture
Primary culture of vascular smooth muscle cells (VSMC) was obtained from aorta explants of WT and RAMP2 TG mice. The VSMC were cultured to third passage in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) containing 20% foetal calf serum (FCS; Sigma). To study the proliferation, the VSMC were trypsinised, placed into 24-well plates at a density of 10^4 cells/well. The cells were quiesced for 24h before addition of drugs. The cells were treated with PBS as control, ATII (10^{-7} M) or ATII (10^{-7} M) + AM_{22-52} (10^{-7} M) in DMEM containing 5% FCS for 24, 48 or 72 hours. Cells were trypsinised and cell counts were performed using haemocytometer.

LPS-induced hypotension and hypothermia
Lipopolysaccharide (LPS, serotype 0127:B8), at a dose equivalent to 2.25x10^6 endotoxin units (EU)/mg in saline (10 ml/kg) was injected i.p. into WT and RAMP2 TG animals. Blood pressure was monitored by tail cuff plethysmography at 1.5 and 4 h after LPS administration. Body temperature was monitored just prior to and 0.5, 1.5 and 4 h after administration of saline or LPS by a rectal probe. The NO_2^-/NO_3^- content of PELF was determined by the Griess assay as an indicator of NO levels. Calcium-independent inducible nitric oxide synthase (iNOS) activity was measured in lung tissue extracts.

Measurement of blood pressure after bolus injection of AM or CGRP
WT and RAMP2 TG mice (25-35 g of matched age and sex), or CD1 mice (25-35 g, female) were anaesthetised with urethane (2.5 g/kg, i.p.) and tracheotomy was performed. The left carotid artery was cannulated and connected to a disposable blood pressure transducer (PowerLab ADInstruments, UK) for blood pressure measurements. The right jugular vein was cannulated for administration of all drugs. Human αCGRP in 0.01% BSA was obtained from Phoenix Pharmaceuticals, USA. Human AM_{1-52}, AM_{22-52} and CGRP_{8-37} were from Bachem, UK and BIBN4096BS was a gift from Boehringer, Germany. Stock solutions were prepared in 10 mM acetic acid and subsequently diluted with sterile saline to indicated concentrations of peptides.

Data analysis and statistics
Data are shown as mean ± SEM. Differences between groups were examined using ANOVA followed by Bonferroni’s or Tukey’s post hoc test. Some studies involving exogenous peptides were analysed using unpaired or paired t-test. p<0.05 was considered statistically significant.
References:


Results

Figure S1

Figure S1: Effects of exogenous intravenous CGRP or AM on mean arterial pressure (MAP) in anaesthetised C57BL6 mice. Baseline blood pressure was 63±5mmHg (n=24). (A) Each CGRP dose was given to the same mouse (depicted by line), increasing doses were given 15 min apart and 0 pmol represents vehicle (0.01% BSA in saline). (B). Each AM dose was given to a separate mouse (depicted by columns), 0 pmol represents the vehicle used. Data was assessed as the maximum % change in MAP from baseline (BL), * p<0.05, ** p<0.01 or *** p<0.001 compared to respective vehicle treated groups; n=4/group.
Figure S2: Effect of dosing with AM (130 pmol/25 g) and/or CGRP (5 pmol/25 g) in anaesthetised CD1 mice. (A) Effect of vehicle given i.v. at 0 min and then AM at 60 min. (B) Effect of AM given i.v. to the same mouse at 0 and then again at 60 min. (C) Effect of vehicle given i.v. at 0 min and then CGRP at 60 min. (D) Effect of CGRP given at 0 and 60 min. (E) Effect of AM given i.v. at 0 min and then CGRP at 60 min. (F) Effect of CGRP given i.v. at 0 min and then AM at 60 min. Data are assessed as the % change in mean arterial pressure (MAP) from baseline (BL). * p<0.05, *** p<0.001 respectively compared to corresponding 1st injection. # p<0.05 compared to AM given after vehicle; n=6-9/group.
Figure S3:
Effect of AM or CGRP in anaesthetised WT (C57BL/6) and RAMP2 TG mice. Baseline blood pressure values of WT and RAMP2 TG mice were 64±2 mmHg and 63±5 mmHg, respectively. (A) Effect of AM (130 pmol/25 g) given i.v. to the same WT mouse at 0 and then again at 60 min. (B) Effect of AM (130 pmol/25 g) given i.v. to the same TG mouse at 0 and 60 min. (C) Effect of CGRP (10 pmol/25 g) given i.v. to the same WT mouse at 0 and then again at 60 min. (D) Effect of CGRP (10 pmol/25 g) given i.v. to the same TG mouse at 0 and then again at 60 min. Data are shown as the % change in mean arterial pressure (MAP) from baseline (BL). * p<0.05 compared to respective 1st injection, n=5-6/group.