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Calcitonin Gene Related Peptide (CGRP) in Autonomic Cardiovascular Regulation and Vascular Structure

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Abstract

Background—CGRP is reported to play important roles in cardiovascular regulation in human and animal models. In spite of this, its role remains controversial. We aim to clarify this by studying the autonomic cardiovascular function and vascular structure in CGRP knockout (CGRP^{-/-}) mice.

Methods—Blood pressure (BP) and heart rate (HR) were assessed by telemeters. Urine (24-hr) and blood were collected for catecholamines measurements. Baroreflex sensitivity was assessed using phenylephrine and sodium nitroprusside administered in an acute study.

Results—Daytime mean arterial blood pressure (MAP, 12-hr period) was significantly higher in the CGRP^{-/-} mice than in the WT mice (114.5 vs. 104.5 mmHg; p=0.04). Norepinephrine was elevated in plasma and 24-hr urine in the knockouts (Urine: 956 vs. 618 pg/ml, p=0.004; Plasma: 2505 vs. 1168 pg/ml, p = 0.04). Paradoxically, cardiovagal baroreflex sensitivity was higher in CGRP^{-/-} mice (3.2 vs. 1.4 ms/mmHg, p=0.03). To increase insight, we studied aortic stiffness in CGRP^{-/-} mice and found it increased compared to age-matched WT mice, as evidenced by the depression of the compliance curve (p<0.05).

Conclusion—CGRP^{-/-} mice have higher BP due to elevated sympathetic signals and abnormalities in blood vessel structure and CGRP plays an important role in the regulation of the cardio-vagal tone.

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Conflict of Interest: None

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Keywords

Baroreflex sensitivity; Catecholamines; sympathetic activities; vagal tone

Background

Calcitonin-gene-related peptide (CGRP) is a 37-amino acid neuropeptide currently viewed as perhaps the most potent known vasodilator [1]. It is 100–1000 times more potent than substance P or acetylcholine [2]. CGRP exists in two forms, α -CGRP and β -CGRP, that share more than 90% homology [3]. While α -CGRP is the result of tissue-specific alternative splicing from the calcitonin gene, β -CGRP is the product of translation from a completely different gene. Both genes are located on chromosome 11 [4]. The majority of CGRP is α -CGRP, which is expressed widely in both central and peripheral nervous systems [5]. In the periphery, α -CGRP is found mainly in sensory neurons that are closely associated with blood vessels, at the junction of the adventitia and the media passing into the muscle layer, where CGRP release is localized appropriately to act as a potential vasodilatory agent [6, 7]. It is also expressed in other organs and tissues important for autonomic cardiovascular regulation, such as the sinoatrial and atrioventricular nodes, *nucleus tractus solitarii*, the vagal nerve and the hypothalamus [8, 9]. The administration of CGRP systemically causes a dose-related decrease in blood pressure (BP) in both normotensive and spontaneously hypertensive rats, as well as in human subjects [10, 11]. Patients with secondary hypertension are reported to have elevated plasma CGRP as a compensatory mechanism for high blood pressure [12]. Interestingly, in essential hypertension subjects, plasma CGRP is reduced compared to healthy volunteers [13, 14]. Studies of BP in CGRP knockout mouse models also have produced discordant results [15, 16]. While Gangula's study showed that mice with α -CGRP/calcitonin knocked out had significantly elevated systolic blood pressure (SBP) and mean arterial blood pressure (MAP) compared to WT mice [15], Lu *et al.* suggested that mice lacking the α -CGRP peptide without the disruption of calcitonin had normal cardiovascular regulation and neuromuscular development at baseline and after exercising [17]. The difference in results might be due to the concurrent deletion of the calcitonin. However, later studies in mice with targeted deletion of only α -CGRP showed that these knockout mice had increased baseline MAP and heart rate (HR) even though calcitonin was intact [16]. These data suggest that CGRP might play an important role in blood pressure regulation in both physiological and pathological conditions, yet its specific function is still debatable.

Given the potential importance of α -CGRP in cardiovascular regulation, the purpose of this study was to characterize hemodynamic and autonomic function of α -CGRP/calcitonin knockout mice. Also, the mechanical properties of blood vessels could contribute significantly to the hemodynamic profile. Therefore, we hypothesized that the lack of α -CGRP/calcitonin in our knockout mice would lessen relaxation of large blood vessels, leading to stiffening of the arteries, decreasing baroreflex sensitivity, and increasing blood pressure.

MATERIALS AND METHODS

All protocols were approved by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC) and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edition, 2011).

Mice

Wild type C57BL/6 mice (n=20; in-house breeding) were used in all of the experiments unless otherwise noted. Global knockout α CGRP/calcitonin mice (*CGRP*^{-/-}; n=19) were generated by Robert F. Gagel, MD from MD Anderson Cancer Center, Texas as previously described [15] and re-derived by Charles River on a C57BL/6 background [18]. Briefly, exons 2 through 5 of the calcitonin I gene were replaced with PGK neoBPA to generate *CGRP*^{-/-} homozygous deficient mice. Animals were 4–5 months and age-matched at the time of all experiments.

Telemeter Implantation

Mice were anesthetized with 4% isoflurane and maintained on 2% isoflurane in oxygen during the surgery. An isothermal pad (Braintree Scientific, Inc.) maintained body temperature at 36°C to 37°C. Before surgery, buprenorphine hydrochloride (4 μ l/g body weight (BW)) and Cefazolin (1.6mg/g BW) were administered intramuscularly. Following aseptic techniques, a telemeter model TA11PA-C10 from Data Science International (DSI) was implanted with the tip of the device inserted into the left carotid artery. A subcutaneous pocket was made on the left side of the body to house the device. Incisions were closed with sterile suture from EthiconTM and the mice given Ringer solution (1ml/25 gram BW) from Baxter, Inc. intraperitoneally. All animals were monitored for at least 1 week before telemetry recording. The telemeter signal was processed using DataQuest ART (Edition 4.3) developed by DSI. The data were continuously sampled at 500Hz processed in 10 sec blocks during day (light on period 06:00–18:00) and night (light off period 18:00–6:00). Analysis was performed when heart rate and blood pressure rhythms normalized after surgery. For statistical analysis, the values for 7 consecutive days were analyzed to assess MAP, HR and activity levels. We obtained the 24hr means as well as 12hr means for the day period and for the night period for each day. Mean MAP, HR and locomotor activities were also calculated for the whole period of seven days. The analysis program was written in MatLab by Dr. Andre' Diedrich, one of the authors.

Urine and Tissue Collection

To collect 24-hr urine, individual mice were placed in a metabolic cage (Fisher). Starting three days before the collection period, the mice were acclimated to the new environment for 6 hours each day. Urine was collected for 24 hours and 6N-HCl (7.5 μ l/1ml urine collected) was added to preserve catechols. Mice were euthanized under isoflurane 3%. Plasma was collected by cardiac puncture and the heart, spleen, adrenal glands, and kidneys were collected and immediately frozen at –80°C for catechol measurement.

Catechol Analysis

Urine and plasma catechols were assayed using the method previously described by Eisenhofer *et al* [19] with variations. Briefly, samples were extracted with alumina, and 3,4-dihydroxybenzylamine was added as an internal standard. Eluate was run on an ESA HPLC system consisting of an ESA 542 Autosampler, ESA pump, Axxi-chrom column (Thompson Instruments, Clearbrook Va.), ESA5011 analytical cell, and Coulochem II detector. Both urine and plasma controls were run in sample sets.

Tissue samples were prepared on ice by adding 0.1N perchloric acid. An Omni International TH homogenizer with disposable probes was used to homogenize the tissues. After homogenization, the samples were sonicated for a minimum of 20 seconds. Finally, they were centrifuged twice at 3000 rpm for 20 minutes; supernatant aliquots were transferred to microfuge tubes and centrifuged at 9500 rpm for 5 min to a clear liquid that was run on the HPLC for catechols.

Baroreflex Sensitivity Assessment

To determine the baroreflex sensitivity in the two genotypes, acute dose response drug studies were done. Mice were under isoflurane anesthesia (1%). Phenylephrine (PHE) and nitroprusside (NTP) were administered through a venous catheter in the left jugular vein. BP was measured through a left femoral artery catheter connected to a pressure transducer (DTX Plus-6), which was then connected to a carrier amplifier to measure heart rate. BP and HR signals were recorded using a WINDAQ data acquisition system (DATAQ) and analyzed by Physiowave software written by Dr. Diedrich in PV-Wave (Visual Numerics). For each drug, 5 different doses (0, 5, 10, 20, 30 and 40 µg/kg) were administered to study the changes in HR in response to change of BP. The sensitivity to PHE and NTP was calculated as the slopes of the dose response curve to each drug using Prism (Graphpad). The baroreflex sensitivity was calculated as the ratio of maximal R-R interval change to the bolus induced MAP change greater than 20 mmHg.

Determination of aortic compliance

Segments of thoracic aorta were mounted onto 0.7 mm cannulas in a pressure-myograph system (Danish Myograph Technologies, Model 110P) and extended to their *in situ* length. Calcium-free buffer was used to eliminate any contribution of active tone of the vessels. A video microscope traced the outer (OD) and inner diameter (ID) of these blood vessels with step-wise increases of intraluminal pressure. Diameters were recorded with every increment of 25 mmHg from 0–200 mmHg. At 200 mmHg, both OD and ID reach a plateau. A vascular compliance curve was constructed by plotting the increment of blood vessel diameters against the corresponding intraluminal pressure applied. A downward shift of the compliance curve indicates aortic stiffening. Stress-strain relationship was also determined [20]. Circumferential stress (σ) was calculated from the following formula:

$$\sigma = (P \times ID) / [2(OD - ID)], P = \text{intraluminal pressure.} \quad \text{Formula 1}$$

Intraluminal pressure was converted from millimeters of mercury to dynes per square centimeter ($1 \text{ mmHg} = 1.334 \times 10^3 \text{ dynes/cm}^2$). Circumferential strain (ϵ) was calculated from the following formula:

$$\epsilon = \Delta OD / OD_0 \quad \text{Formula 2}$$

OD_0 is the original outer diameter, defined as diameter at 0 mmHg. OD was calculated as the increase of outer diameter from OD_0 at each applied pressure.

Measurement of aortic collagen and elastin

Masson's trichrome staining and Verhoeff staining were used to visualize aortic collagen and elastin respectively. In separate samples, aortic collagen was quantified by measurement of tissue hydroxyproline as previously described [21]. In additional vessels, elastin content was determined as described by Mecham et al., with modifications [22, 23]. Briefly, insoluble elastin was separated from all other soluble proteins by hydrolysis in 0.1N NaOH at 90°C for 45 minutes. Both the soluble and insoluble fractions were further digested in 6N HCl at 105°C for 48 hours, neutralized and assayed for ninhydrin content.

Statistics

Data are presented as mean $\pm$ SE. To assess genotype differences in hemodynamic variables, we used 2-way ANOVA for repeated measurements. Tests of the change in BP in response to treatment within each treatment group were assessed by comparing BP and HR using a Mann-Whitney U test. The Mann-Whitney test was also used to assess differences in catechols. The Mann-Whitney U test was chosen to eliminate any potential of unusual outliers. Genotype-related differences in aortic stiffening were evaluated by two-way ANOVA. Statistical significance was accepted when $P < 0.05$.

RESULTS

Effects of α -CGRP/calcitonin gene deletion on body weight, tissue weights and activity levels

CGRP^{-/-} mice weighed significantly more than age-matching WT mice (CGRP^{-/-}: 31.9 ± 4.1 vs. WT: 26.6 ± 1.4 grams, $n=16$, $p<0.0001$) (Figure S1A). These mice also tended to be less active, indicated by the lower 24-hr locomotor activity data from the telemetry (CGRP^{-/-}: 6.6 ± 3.5 vs. 9.0 ± 2.7 counts/minute, $n=9$, $p=0.11$) (Figure S1B). To assess the possibility of organ damage such as hypertrophy, we measured the weights of the heart, kidneys and spleen and normalized them to body weight (Figure S3). There was no significant difference between the two genotypes. No difference in feeding behaviors was observed between the two groups.

Effect of α -CGRP/calcitonin gene deletion on blood pressure and heart rate

There was a trend for higher MAP in the CGRP^{-/-} mice, but this was not significant at any individual 12-hour data point (Figure 1A). However, the 7-day average daytime MAP was significantly higher in CGRP^{-/-} mice (CGRP^{-/-}: 114.5 ± 10.6 versus WT: 104.5 ± 5.5

mmHg; $P=0.04$; Figure 1B). Seven-day average nighttime MAP showed a trend for being higher in CGRP^{-/-} mice (CGRP^{-/-}: 128.0 ± 12.5 versus WT: 119.7 ± 5.9 mmHg, $P=0.09$) (Figure 1B). There was no difference in HR between the two genotypes, whether compared for individual 12-hour periods (Figure 1C) or 12-hour periods averaged over seven days (Figure 1D). Similarly, the 24-hr average MAP, systolic blood pressure (SYS), diastolic blood pressure (DIA) and HR calculated from the seven days of data showed no significant differences between the two groups (MAP: CGRP^{-/-}: 120.1 ± 10.2 versus WT: 112.9 ± 5.8 mmHg, $n=9$, $p=0.25$; HR: CGRP^{-/-}: 561.1 ± 36.4 versus WT: 579.8 ± 27.8 bpm, $n=9$, $p=0.19$) (Figure S2 and Table 1).

Catecholamine profiles of CGRP^{-/-} mice

Urine—For CGRP^{-/-} mice, urinary norepinephrine was significantly higher than for their WT counterparts (CGRP^{-/-}: 956 ± 90 pg/ml, $n=9$ versus WT: 618 ± 46 pg/ml, $n=9$, $p=0.0007$). Dihydroxyphenylglycol (DHPG), an intraneuronal metabolite of norepinephrine which can reflect reuptake through the norepinephrine transporter, was also higher in the knockout mouse group (CGRP^{-/-}: 603 ± 84 pg/ml, $n=9$ versus WT: 377 ± 31 pg/ml, $n=9$, $p=0.02$) (Figure 2A). The ratio of NE/DHPG was not different (CGRP^{-/-}: 0.62 ± 0.03 , $n=9$ versus WT: 0.62 ± 0.04 , $n=9$, $p=0.84$) (Figure 2B). Epinephrine and dopamine levels did not differ between genotypes (Table 2).

Plasma—As was the case in urine, plasma norepinephrine was significantly higher in CGRP^{-/-} compared to WT mice (CGRP^{-/-}: 2505 ± 596 pg/ml, $n=6$ versus WT: 1168 ± 98 pg/ml, $n=7$, $p=0.02$). Plasma DHPG was not significantly higher in CGRP^{-/-} mice (CGRP^{-/-}: 4626 ± 670 pg/ml, $n=6$ versus WT: 3347 ± 526 pg/ml, $n=7$, $p=0.2$) (Figure 2C). The ratio of NE/DHPG was also not different between the two groups (CGRP^{-/-}: 0.82 ± 0.35 , $n=6$ versus WT: 0.42 ± 0.09 , $n=7$, $p=0.53$) (Figure 2D). Epinephrine was significantly higher in CGRP^{-/-} than in WT mice (Table 2). There was no difference in plasma dopamine between CGRP^{-/-} and WT mice (Table 2).

Tissues—To measure catecholamines in the tissues, mice were placed under isoflurane 2% for at least 15 minutes before tissue retrieval. There were no significant differences in tissue catechol levels between the CGRP^{-/-} and the WT mice (Table 2).

Effect of α -CGRP/calcitonin gene deletion on baroreflex sensitivity

To construct the dose response curves for PHE and NTP, five doses (0 – 40 ug/kg), were given in random order to each WT and CGRP^{-/-} mouse. The baroreflex sensitivity (BRS) was calculated at the 40ug/Kg dose which produced a BP change greater than 20 mmHg MAP without causing arrhythmia. As indicated by similar BP dose-response slopes (WT: 0.66 ± 0.09 versus CGRP^{-/-}: 0.59 ± 0.11 , $P=0.64$), the two genotypes did not differ in sensitivity to PHE or NTP (Figure 3A and 3B). CGRP^{-/-} mice had significantly higher baroreflex sensitivity compared to their WT counterparts when assessed with PHE (CGRP^{-/-}: 3.21 ± 0.46 versus WT: 1.40 ± 0.26 ms/mmHg, $p=0.026$; Figure 3C). However, no differences in baroreflex sensitivity were observed using NTP boluses (Figure 3D).

Blood vessel stiffening in CGRP^{-/-} mice

To investigate the passive mechanical structure of the vasculature, thoracic aortas were collected and the increase in vessel diameters measured as intraluminal pressure was raised. In the physiological range of blood pressure (100–175 mmHg), the compliance curve for the CGRP^{-/-} mice was markedly depressed compared to that of WT mice (Figure 4A). Similarly, a leftward shift of the stress-strain curve was observed in CGRP^{-/-} mice, also indicative of aortic stiffening (Figure 4B).

To determine whether the stiffer blood vessels in CGRP^{-/-} mice were related to a difference in collagen and elastin composition, the thoracic aortas were subjected to biochemical assays to quantify elastin and collagen content. Surprisingly, no difference in elastin or collagen content was seen between CGRP^{-/-} and WT aortas (Figure 4C). Histological staining of collagen and elastin revealed similar distribution and content of elastin and collagen in the thoracic aortas. (Figure 4D).

DISCUSSION

There is a lot of evidence to suggest the involvement of CGRP in the cardiovascular system, but there is not yet agreement on how this peptide affects BP. The primary findings of our study are that CGRP^{-/-} mice have: 1) elevated daytime MAP; 2) higher urinary and plasma norepinephrine concentrations; 3) increased baroreflex-mediated vagal activation after α adrenergic receptor stimulation and 4) significant aorta stiffening.

CGRP is extensively distributed in the central and peripheral nervous systems and in the cardiovascular system. It is mainly synthesized in the dorsal root ganglion (DRG), which contains sensory neurons [24]. These neurons terminate on blood vessels and when CGRP is released in the periphery, it exerts a very potent vasodilator effect, either via the release of nitric oxide from the endothelial cells or directly through the relaxation of the smooth muscle cells. When exogenous CGRP is administered systemically, BP decreases in a dose-dependent manner [10, 24–26]. CGRP expression in the DRG is decreased in Dahl salt-sensitive hypertensive and spontaneously hypertensive rats [27, 28].

Our data showed that age-matched CGRP^{-/-} mice had higher body weight compared to the WT mice. The ratios of tissues to body weight for the heart, kidneys and spleen were not different between the two genotypes, suggesting a lack of hypertrophy (Figure S3). However, hypertensive organ damage cannot be ruled out without future histological and functional studies. CGRP^{-/-} mice have been consistently reported to have higher bone mass density and up to 2-fold higher bone volume [29–33]. This might explain the increase in body weight of these knockout mice. Further studies of body composition would give a more definite explanation for this interesting observation.

Our finding of elevated BP in CGRP^{-/-} mice is consistent with a report by Gangula *et al.* using tail cuff blood pressure measurements to show that α CGRP/calcitonin knockout mice are hypertensive [15]. This observation is also similar to Oh-hashii's blood pressure data in which BP was recorded via a catheter inserted into the femoral artery in α CGRP specific knockout mice [34]. In the study by Lu *et al.*, which found no difference in BP in α CGRP

specific-knockout mice, blood pressure was assessed using a catheter inserted into the carotid artery and recorded for two minutes of integration time [17]. This discrepancy in findings is not due to the additional knockout of the calcitonin but might be related to a difference in the method for blood pressure measurement and the time period of recording. Our study used telemetry, which allowed continuous beat-to-beat measurement of BP during a 7-day period. A difference between genotypes emerged in our studies only when MAP data were averaged over the seven days, and this reached significance only for daytime pressures. In Table 1, we noted that daytime systolic and diastolic blood pressures showed a trend toward higher values in CGRP^{-/-} mice (p-value = 0.06 and 0.05, respectively). Therefore, the effect on the BP of the CGRP^{-/-} mice may be subtle and masked by the variability of BP. As a result, caution needs to be taken to collect data continuously and over a longer period when assessing blood pressure in the CGRP^{-/-} mice.

There might be several mechanisms and contributors that explain this elevation in BP. First, plasma and urine norepinephrine were elevated in CGRP^{-/-} mice. Norepinephrine can be released from the adrenal medulla into the blood as a hormone or as a neurotransmitter from sympathetic noradrenergic neurons. Significantly and sustained elevation of plasma norepinephrine to levels above 1000 pg/ml is frequently associated with sustained increases in blood pressure in man [35], in patients with pulmonary hypertension and also in animals [36] [35, 37–40]. However, it is noteworthy that the effect of norepinephrine depends on its origin. A plasma level of 1000 pg/ml by infusion of low doses of norepinephrine into a human subject might have little effect on BP, whereas endogenously released norepinephrine stimulated by vascular neuronal norepinephrine release mechanisms would generally produce a mild pressor effect at this level. Though not a perfect marker, plasma norepinephrine elevation does reflect an increase in sympathetic activity when samples are carefully obtained as in these CGRP^{-/-} mice. Even though the cardiac puncture method used to collect blood might raise the catecholamine levels, all mice were acclimated in isoflurane 1% for 20 minutes before the procedure. As shown in Figure 2, 24-hr urine norepinephrine concentration was also higher in CGRP^{-/-} mice, indicating increased time-integrated sympathetic activity and a normal urinary clearance mechanism. Plasma norepinephrine reuptake was not different, reflected by similar NE/DHPG ratios (Figure 2). Interestingly, CGRP has been reported to inhibit norepinephrine release in rat hypothalamus [35]. Therefore, it is possible that the lack of CGRP could release this inhibitory mechanism leading to an increase in plasma NE. However, norepinephrine synthesis or metabolism might also be altered in the CGRP^{-/-} mice. Sympathetic input to the heart is the primary stimulator of cardiac contractility in mice [41]. Our data showed that norepinephrine had a trend to be elevated in the heart tissues of CGRP^{-/-} mice (Table 2, p=0.09). This could lead to increased stimulation of the hearts in the CGRP^{-/-} mice and therefore, increased blood pressure. Secondly, research by Li *et al.* showed that the renin-angiotensin system (RAS) is activated in α CGRP/calcitonin knockout mice [42]. The activation of RAS has been well documented to raise BP in both humans and animals [43–47] and might be contributory to the increase in blood pressure in these CGRP^{-/-} mice as well.

The arterial baroreflex is the most relevant factor in adapting to acute changes in pressure [48]. It senses mechanical stretch and chemical changes in the blood to either activate or

reduce sympathetic tone to maintain a relatively constant blood pressure. The sensitivity of the baroreflex reflects its ability to control HR and BP variability [49, 50]. Elevated plasma norepinephrine is strongly correlated with decreased baroreflex sensitivity in normal and pathological states [51–53]. Contrary to what we expected, baroreflex sensitivity for HR control was improved in the knockout mice, with selective improvement in the vagal arm of the reflex as indicated by the baroreflex sensitivity to phenylephrine (Figure 3C).

This elevated BRS was not due to increased sensitivity of α_1 adrenergic receptors since the blood pressure-dose response relationship for phenylephrine was similar for WT and CGRP^{-/-} mice (Figure 3). Interestingly, the enhanced baroreflex sensitivity is consistent with findings in mice that have overexpressed human receptor activity-modifying protein (hRAMP), a component of the receptor complex that binds strongly to CGRP[54]. It is possible that CGRP^{-/-} mice have an elevated expression or activity of RAMP as a compensatory mechanism for the loss of the peptide.

Our data showed also that α_1 adrenergic receptor sensitivity and sensitivity to NO are not altered in the knockout mice (Figure 3). We also showed that the vessels are stiffer which could cause an increase in blood pressure (Figure 4). This could stimulate baroreflex-mediated vagal activity to counteract increases in blood pressure. An increase in vagal activity is consistent with the trend toward a lower heart rate in the CGRP knockout mice (Figure 1 and Figure S2). Other ways in which CGRP depletion could enhance the baroreflex-mediated fall in heart rate after phenylephrine include an effect on the afferent signal of the baroreflex, such as increased responsiveness of the baroreceptors, or an effect in the brainstem that causes a change in the baroreflex sensitivity curve. As a result, the vagal increase/sympathetic withdrawal response to a vasopressor agent is enhanced. Masuki et al. described enhanced baroreflex sensitivity in calponin knockout mice [55]. The calponin knockout mice had reduced elasticity and an enhanced response to phenylephrine, but no change in the response to nitric oxide, a similar result to that presented in this paper. Direct measurement of sympathetic activity would provide more information that could help explain this paradoxical increase in baroreflex sensitivity.

Aortic stiffening has been shown to be associated with hypertension in humans and in animal models [54, 56]. CGRP^{-/-} mice had significant stiffening in the thoracic aorta without changes in collagen/elastin composition or distribution. The vascular compliance studies were performed in calcium-free buffer to eliminate vascular tone; therefore, vasoconstriction could not have contributed significantly to aortic stiffening in this model. CGRP released from damaged dorsal root ganglia suppresses the expression of transglutaminase-1 and transglutaminase-3 that form extensively cross-linked covalent bonds among matrix proteins [57]. Increased expression or activity of tissue transglutaminase causes protein cross-linking and aortic stiffening in hypertension [56, 58]. In addition, the deficiency of α -CGRP in DOCA-salt hypertensive mice increases isoprostane excretion, which could also stimulate protein cross-linking [59]. Therefore, it is possible that protein cross-linking contributes to aortic stiffening in CGRP^{-/-} mice. An alternative explanation for aortic stiffening relates to the sympathetic activation indicated by high levels of norepinephrine in both plasma and urine of the CGRP^{-/-} mice (Figure 2 and table 1). Wang *et al.* injected mice with recombinant leptin and showed that chronic

activation of the sympathetic nervous system and superoxide production likely play a role in mediating the effect of leptin to cause endothelial dysfunction with subsequent aortic stiffness [60]. Therefore, it is possible that the lack of CGRP in the knockout mice caused a prolonged sympathetic activation, which led to stiffening of the blood vessels. Recently, it was suggested that smooth muscle cell stiffening might be a key contributor to aortic stiffness [61]. CGRP inhibits the proliferation of vascular smooth muscle cells [62], therefore disrupting the normal change in remodeling and reorganizing of these cells in blood vessels. Aortic stiffness has been found to precede hypertension both in animal models and in large population cohorts [63, 64]. Future studies are required on the mechanisms that can lead to the stiffening of blood vessels in these mice.

PERSPECTIVE

The role of CGRP in blood pressure regulation remains controversial. Given the abundant expression of this peptide in the nervous system, its powerful vasodilatory effect, and the increasing number of reports on its contribution to cardiovascular disease, it is important to understand its role. Our data showed that α CGRP/calcitonin knockout mice have significantly higher blood pressure, as reflected during telemetry monitoring over a seven-day period. This difference in BP is subtle and can be missed if insufficient data is collected. The association of CGRP absence with elevation of sympathetic nervous system activity, RAS activation and aortic stiffening in the pre-hypertensive system suggests that CGRP agonists might have effects to reduce blood pressure. These mice have elevated norepinephrine levels, enhanced baroreflex sensitivity, and aortic stiffening. Therefore, therapeutic use of CGRP antagonists in proposed treatment of migraine should take into account these effects that might accompany the loss of CGRP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Mean arterial blood pressure (MAP) and heart rate (HR) in WT and CGRP^{-/-} mice. A) MAP in daily 12-hour periods B) MAP in 7-day period C) HR in 12-hour periods D) HR in 7-day period. (* P=0.04, n=9 in each group)

Figure 2.

24-hr urine and plasma norepinephrine levels. A) Norepinephrine concentration in 24-hr urine was significantly higher in CGRP^{-/-} mice compared to WT mice ([#]P=0.004, n=9 in each group). B) NE/DHPG ratio in 24-hr urine C) Plasma norepinephrine was also higher in CGRP^{-/-} mice compared to WT mice (* P=0.035, n=6 for CGRP^{-/-} and n=7 for WT) D) NE/DHPG in plasma.

Figure 3.

Sensitivity to Phenylephrine and Nitroprusside in WT and CGRP^{-/-} mice. A) Phenylephrine dose response curves. B) Nitroprusside dose response curves. C) Baroreflex sensitivity calculated at 40 μ g/kg of Phenylephrine, representing the vagal activation and sympathetic withdrawal (*P=0.02). D) Baroreflex sensitivity calculated at 40 μ g/Kg of Nitroprusside, representing vagal withdrawal and sympathetic activation.

Figure 4.

Blood vessel stiffening in CGRP^{-/-} mice. A) Compliance curve (n=5 in each group, #P<0.01). B) Stress-strain curve (n=5 in each group) C) Quantification of collagen (hydroxyproline/total protein), elastin and the ratio of elastin/collagen in thoracic aortas (n=5 for each group). D) Staining for collagen (trichrome blue) and elastin in WT and CGRP^{-/-} mice.

Table 1

Blood Pressure Values in CGRP^{-/-} and WT mice recorded by telemeters.

BP (mmHg)	WT	CGRP ^{-/-}	p
24hr-SYS	129.8 ± 7.6	135.3 ± 8.2	0.19
24hr-DIA	96.1 ± 5.7	106.5 ± 15.3	0.14
Nighttime SYS	138.8 ± 7.6	142.3 ± 9.7	0.67
Nighttime DIA	103.7 ± 7.1	112.4 ± 17.3	0.49
Daytime SYS	120.9 ± 7.9	128.2 ± 7.8	0.06
Daytime DIA	88.5 ± 4.8	100.5 ± 13.8	0.05

Table 2

Tissue Catecholamines

Catecholamines	Norepinephrine			Epinephrine			Dopamine		
	WT	CGRP ^{-/-}	p	WT	CGRP ^{-/-}	p	WT	CGRP ^{-/-}	p
Heart (ng/g)	735 ± 35	825 ± 34	0.09	23.3 ± 3.9	11.8 ± 3.2	0.05	37.7 ± 4.4	36.7 ± 3.1	0.84
Spleen (ng/g)	1229 ± 107	1097 ± 123	0.43	18.0 ± 1.5	10.9 ± 4.6	0.19	86.9 ± 12.2	78.8 ± 14.1	0.68
Left Kidney (ng/g)	437 ± 16	417 ± 24	0.5	366 ± 89	429 ± 177	0.74	61.0 ± 7.7	62.7 ± 8.3	0.87
Right Kidney (ng/g)	458 ± 30	455 ± 212	0.96	350 ± 89	370 ± 207	0.57	60.6 ± 7.2	62.9 ± 7.8	0.83
Plasma (pg/ml)	1168 ± 98	2505 ± 596	0.035	359 ± 128	525 ± 97	0.035	N/A	N/A	
24-hr Urine (pg/ml)	618 ± 46	956 ± 90	0.004	83.6 ± 12.4	103 ± 9	0.2	974 ± 98	1100 ± 122	0.44

N/A= Not Detectable