Vascular reactivity to calcitonin gene-related peptide is enhanced in subtotal nephrectomy-salt induced hypertension

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Supowit SC, Katki KA, Hein TW, Gupta P, Kuo L, Dickerson IM, DiPette DJ. Vascular reactivity to calcitonin gene-related peptide is enhanced in subtotal nephrectomy-salt induced hypertension. Am J Physiol Heart Circ Physiol 301: H683–H688, 2011. Published June 10, 2011; doi:10.1152/ajpheart.00598.2009.—In subtotal nephrectomy (SN)- and salt-induced hypertension, calcitonin gene-related peptide (CGRP) plays a compensatory role to attenuate the blood pressure increase. In the absence of an increase in the neuronal synthesis and release of this peptide. Therefore, the purpose of this study was to determine whether the mechanism of this antihypertensive activity is through enhanced sensitivity of the vasculature to the dilator actions of this neuropeptide. Hypertension was induced in Sprague-Dawley rats by SN and 1% saline drinking water. Control rats were sham-operated and given tap water to drink. After 11 days, rats had intravenous (drug administration) and arterial (continuous pressure recording) catheters surgically placed and were studied in a conscious restrained state. Baseline mean arterial pressure was higher in the SN-salt rats (157 ± 5 mmHg) compared with controls (128 ± 3 mmHg). Administration of CGRP (and adrenomedullin) produced a significantly greater dose-dependent decrease in mean arterial pressure in SN-salt rats compared with controls (~2.0-fold for both the low and high doses). Interestingly, isolated superior mesenteric arterioles from SN-salt rats were significantly more responsive to the dilator effects of CGRP (but not adrenomedullin) than the controls (pEC50, SN-salt, 14.0 ± 0.1 vs. control, 12.0 ± 0.1). Analysis of the CGRP receptor proteins showed that only the receptor component protein was increased significantly in arterioles from SN-salt rats. These data indicate that the compensatory antihypertensive effects of CGRP result from an increased sensitivity of the vasculature to dilator activity of this peptide. The mechanism may be via the upregulation of receptor component protein, thereby providing a more efficient coupling of the receptor to the signal transduction pathways.

This study suggests that the vasculature might be more responsive to the vasodilator activity of CGRP and perhaps AM. Therefore, the purpose of this current study was to determine if there is a significant in vivo increase in vascular reactivity to CGRP or AM in SN-salt hypertension and to determine if this hyperresponsiveness could be recapitulated in isolated arterial preparations. Expression of CGRP receptor.

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proteins was monitored in parallel with the physiological studies to determine if there was a relationship between receptor protein expression and CGRP efficacy.

METHODS

Animals. Animal protocols were approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN), initially weighing 150 g, were used for this study. For the surgical procedures, the rats were anesthetized with ketamine and xylazine (80 and 4 mg/kg ip). SN-salt hypertension was induced by the removal of the right kidney and ~25% of the left kidney (17). Animals received 1% saline to drink. Sham-operated animals that received tap water were used as controls. The tail-cuff method (Narco Bio Systems, Austin, TX) was used to record blood pressures before the surgeries and every 3 days thereafter. The animals were studied 11 days after the surgical procedure.

Mean blood pressure determinations. The rats that received exogenous CGRP or AM were anesthetized as described above. The left carotid artery was cannulated for continuous measurement of mean arterial pressure (MAP) and heart rate with a pressure transducer linked to a recorder (Gould Instruments, Valley View, OH; see Ref. 16). The right jugular vein was also cannulated for infusion of either vehicle (saline), CGRP, or AM (American Peptide, Sunnyvale, CA). Hemodynamic studies were performed ~3 h after surgery, with the rats fully awake and unrestrained. Values for the observed blood pressure changes following CGRP or AM administration were determined from the integrated areas under the curve. These values were then normalized to the baseline MAP of either the SN-salt or control groups.

Functional assessment of isolated mesenteric arterioles. For these studies, additional groups of SN-salt (n = 10) and control (n = 11) rats were used. The tail-cuff method was used to determine blood pressures before initiation of the SN-salt protocol and every 3 days thereafter as described above. After 11 days, the SN-salt hypertensive and control rats were anesthetized, and the small intestine was removed. Third-order arterioles (~1 mm in length; 40–60 μm internal diameter) were isolated from the mesentery. The arteriole was then transferred to a chamber containing physiological salt solution (PSS) with 1% albumin and equilibrated at ambient temperature. Both ends of the arteriole were then cannulated using glass micropipettes filled with PSS-albumin solution (10). The vessel was transferred to the stage of an inverted microscope coupled to a video camera and video micrometer for continuous measurement of the internal diameter. The micropipettes were connected to pressure reservoirs, and the vessel was pressurized to 60 cm H2O intraluminal pressure without flow. Arterioles were bathed in PSS-albumin at 36–37°C to allow development of basal tone (~60 min). The concentration-dependent responses to CGRP (0.1 nM to 10 nM; American Peptide), AM (0.1 μM to 1 μM; American Peptide), or sodium nitroprusside (SNP; 1 nM to 0.1 mM; Sigma-Aldrich, St. Louis, MO) were examined. In some vessels, dilation in response to CGRP and SNP was evaluated in the absence and presence (30-min pretreatment) of the CGRP receptor antagonist CGRP8–37 (1 μM; American Peptide). Arterioles were exposed to each dose of the vasodilator agents for 1–2 min until a stable diameter was established. At the end of each experiment, the vessel was relaxed with 0.1 mM SNP to obtain its maximal diameter at 60 cm H2O intraluminal pressure. Diameter changes in response to agonists were normalized to this maximal dilation and expressed as a percentage of maximal dilation (10). Data are reported as means ± SE. The EC50 values are presented as the negative logarithm (pEC50) and calculated using GraphPad Prism software.

Western blot analysis. The RAMP1, RCP, and CLR antibodies were provided by one of the authors (Dickerson; see Refs. 4 and 14). RAMP1 is a rabbit polyclonal antibody against the synthetic peptide MVTACRDPPDYGT of mouse RAMP1. RCP is a chicken polyclonal antibody against mouse RCP sequence EEQQEALHTVT conjugated to keyhole limpet hemocyanin. CLR is a rabbit polyclonal antibody against the mouse CLR sequence GYSHDCPTEHLNGK. The RAMP2 and RAMP3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the anti-actin antibody was obtained from Sigma Aldrich. For this series of experiments, separate groups of SN-salt (n = 12) and control (n = 12) were used. Again, systolic blood pressures were determined by the tail-cuff method as described previously. Superior mesenteric arteries from SN-salt and control rats were isolated and frozen. Protein content of the membrane preparations was measured by the Bradford method (Bio-Rad Laboratories, Hercules, CA), and equal amounts of cell lysates were subjected to 12.5% SDS-PAGE and transferred to membranes. Each sample was analyzed a minimum of three times. Membranes were incubated for 3 h with the indicated antiserum, washed, and then incubated again with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences, Arlington Heights, IL). Immunocomplexes were then visualized using chemiluminescence detection. For all of the blots, the linearity of the chemoluminescence signal with input protein was verified.

Statistical analysis. Statistical comparisons of data were performed by Student’s t-test or by two-way ANOVA followed by the Bonferroni multiple-range test, as appropriate. A value of P < 0.05 was considered significant.

RESULTS

Effects of CGRP and AM on blood pressure. Systolic blood pressures were determined by the tail-cuff method before surgery and every three days thereafter. The average systolic blood pressures were 210 ± 11 and 143 ± 4 mmHg for the SN-salt and control animals, respectively. For the in vivo dose-response studies, separate groups of SN-salt (n = 6) and control (n = 6) rats were used for each dose of agonist. On day 11 after initiation of the SN-salt protocol, rats had arterial (for continuous MAP recording) and intravenous (for drug administration) catheters surgically implanted and were studied in a fully awake and unrestrained state. The changes in MAP were determined by calculating the integrated areas under the curve. Because the basal MAP levels were different between the SN-salt (157 ± 5 mmHg) and control (128 ± 3 mmHg) animals, it was necessary to normalize the values obtained for the CGRP- and AM-induced MAP decreases to the basal MAPs. Therefore, the data in Figs. 1 and 2 are expressed as a percentage decrease in baseline MAP. Administration of saline (0.1 ml iv) did not significantly change MAP in either group (data not shown). In contrast, administration of a bolus dose of CGRP (0.128 nM/kg) produced a 9.2 ± 2.6% decrease in MAP (11 mmHg maximum reduction) in the control rats and an 18.3 ± 1.7% MAP decrease (27 mmHg maximum reduction) in the hypertensive animals (Fig. 1). Likewise, the higher 0.64 nM/kg dose of CGRP produced a 17.5 ± 1.0% MAP decrease (20 mmHg maximum reduction) in the control rats and a 25 ± 2.5% MAP decrease (40 mmHg maximum reduction) in the hypertensive animals. The depressor effects of CGRP (and AM) occurred rapidly (10–15 s) and lasted for ~15–20 and 30–35 min for the low and high doses, respectively. Similar results were obtained following administration of AM (Fig. 2). The lower dose of AM (0.5 nM/kg) resulted in a 5.1 ± 0.4% decrease in MAP (8 mmHg maximum reduction) in the control rats and a 12.2 ± 1.0% MAP decrease (21 mmHg maximum reduction) in the hypertensive animals. The 1 nM/kg dose produced a 9.3 ± 0.4% decrease in MAP (15 mmHg maximum reduction) in the control rats and a 21.2 ± 1.7% baseline
decrease in MAP (32 mmHg maximum reduction) in the SN-salt rats.

Effect of CGRP and AM on mesenteric arterioles. Mesenteric arterioles from SN-salt (n = 10) and control (n = 11) rats were isolated and pressurized without flow. There were no significant differences in resting diameter (control, 58 ± 3 vs. SN-salt, 58 ± 4 μm), maximal diameter (control, 92 ± 6 vs. SN-salt, 98 ± 5 μm), and basal tone (control, 64 ± 3% vs. SN-salt, 58 ± 2%) in the vessels from the two groups. Treatment of the arterioles from control and SN-salt rats with CGRP produced concentration-dependent dilations (Fig. 3A); however, the arterioles from the SN-salt rats displayed a marked leftward shift of the curve, indicating a significant increase in vascular reactivity to CGRP (pEC50, SN-salt, 14.0 ± 0.1 vs. control, 12.0 ± 0.1). To ensure that the effects of CGRP were mediated through the CGRP receptor, a concentration-response curve was generated using arterioles from both groups in the presence of the CGRP receptor antagonist CGRP8–37. The antagonist inhibited the dilator activity of vessels from the SN-salt (Fig. 3B) and control (data not shown) rats in response to CGRP without significantly altering the response to the highest concentration (10 nM) of CGRP. In contrast, the mesenteric arterioles from both groups were markedly less responsive to AM than to CGRP, and the concentration-dependent dilation curves were virtually identical between the two groups (Fig. 4).

To determine whether the arteriole preparations would respond differently to a receptor-independent vasodilator, both were treated with increasing concentrations of the nitric oxide donor SNP. As shown in Fig. 5A, the concentration-response curves were similar (pEC50, SN-salt, 7.0 ± 0.2 vs. control, 7.1 ± 0.2), and treatment of SN-salt vessels with CGRP8–37 did not alter vasodilation to SNP, indicating that the CGRP antagonist did not exert a nonspecific inhibitory effect on vascular reactivity (Fig. 5B). Similar results were obtained when the arterioles from the control rats were treated with the CGRP receptor antagonist (data not shown).

Western blot analysis of the CGRP/AM receptor. The final series of experiments used Western blot analysis of mesenteric arterioles from SN-salt hypertensive rats are hyperresponsive to CGRP. A: mesenteric arterioles isolated and pressurized without flows from SN-salt (○, n = 10) and control (●, n = 11) rats were treated with increasing concentrations of CGRP. B: arterial preparations from SN-salt rats (n = 6) were treated with increasing concentrations of CGRP in the absence (●) and presence (○) of CGRP8–37. Values shown are means ± SE. *P < 0.05, SN-salt vs. control (A) and control in the presence of CGRP8–37 vs. control in the absence of the antagonist (B).

Fig. 1. The mean arterial pressure (MAP) of subtotal nephrectomy (SN)-salt hypertensive rats is more sensitive to exogenous calcitonin gene-related peptide (CGRP). Groups (n = 6) of SN-salt and control rats were instrumented for MAP recording and CGRP administration as described in the text. With the rats fully awake and unrestrained, bolus doses of the indicated amounts of CGRP were given intravenously (0.1 ml in saline). Percent baseline MAP values are reported as means ± SE. **P < 0.01, SN-salt vs. control rats at the lower CGRP dose; SN-salt vs. control rats at the higher CGRP dose. *P < 0.05, SN-salt rats at the higher CGRP dose vs. SN-salt rats at the lower CGRP dose.

Fig. 2. The MAP of SN-salt hypertensive rats is more sensitive to exogenous adrenomedullin (AM). Groups (n = 6) of SN-salt and control rats were instrumented for MAP recording and AM administration as described in the text. With the rats fully awake and unrestrained, bolus doses of the indicated amounts of AM were given intravenously (0.1 ml in saline). Percent baseline MAP values are reported as means ± SE. **P < 0.01, SN-salt vs. control rats at the lower AM dose; SN-salt vs. control rats at the higher AM dose; SN-salt rats at the higher AM dose vs. SN-salt rats at the lower AM dose.

Fig. 3. Isolated mesenteric arterioles from SN-salt hypertensive rats are hyperresponsive to CGRP. A: mesenteric arterioles isolated and pressurized without flows from SN-salt (○, n = 10) and control (●, n = 11) rats were treated with increasing concentrations of CGRP. B: arterial preparations from SN-salt rats (n = 6) were treated with increasing concentrations of CGRP in the absence (●) and presence (○) of CGRP8–37. Values shown are means ± SE. *P < 0.05, SN-salt vs. control (A) and control in the presence of CGRP8–37 vs. control in the absence of the antagonist (B).

Fig. 4. To determine whether the arteriole preparations would respond differently to a receptor-independent vasodilator, both were treated with increasing concentrations of the nitric oxide donor SNP. As shown in Fig. 5A, the concentration-response curves were similar (pEC50, SN-salt, 7.0 ± 0.2 vs. control, 7.1 ± 0.2), and treatment of SN-salt vessels with CGRP8–37 did not alter vasodilation to SNP, indicating that the CGRP antagonist did not exert a nonspecific inhibitory effect on vascular reactivity (Fig. 5B).
artery membrane preparations from control (n = 12) and SN-salt (n = 12) animals to determine whether the increase in vascular reactivity to CGRP (and AM) in SN-salt hypertension was associated with alterations in the levels of one or more of the components of the CGRP/AM receptor. Figure 6 is a representative composite blot that indicated that there were no significant alterations in the levels of RAMPs 1–3 between the SN-salt and control groups. Figure 7 is a representative composite blot comparing the levels of CLR and RCP between the two groups. CLR content was unchanged in the SN-salt arterial preparations compared with controls. In contrast, the expression of RCP was increased 1.4-fold in the hypertensive rats.

DISCUSSION

This paper reports the following three new observations: 1) the in vivo hypotensive effects of exogenous CGRP and AM were significantly greater in SN-salt hypertensive rats than in controls; 2) the in vitro vascular reactivity of mesenteric arterioles to CGRP, but not AM, was enhanced significantly in SN-salt hypertension; and 3) RCP content was increased significantly in the SN-salt arterioles in the absence of any changes in CLR or RAMPs 1–3. These findings are supported by other studies showing that CGRP (and AM) is a potent regulator of smooth muscle (vascular, uterine, gastrointestinal tract) function (4, 7, 12, 14, 18, 20). These activities are regulated at the level of the vasculature via mechanisms that are, in most cases, correlated with alterations in the CGRP receptor (7, 9, 11, 12, 15, 18, 20). For example, alterations in CGRP potency in the absence of any changes in CGRP receptor number have been reported. Using isolated hypoxic porcine intramyocardial arteries, Hasbak et al. (7) demonstrated increased affinity of the receptor for CGRP, increased vascular cAMP, and enhanced vasorelaxant effects of CGRP, but not AM, without any changes in CLR or RAMP1 and

Fig. 4. Isolated mesenteric arterioles from SN-salt hypertensive rats are not hyperresponsive to AM. Mesenteric arterioles isolated and pressurized without flow from SN-salt (○, n = 7) and control (●, n = 8) rats were treated with increasing concentrations of AM. Values shown are means ± SE.

Fig. 5. SN-salt hypertension does not globally increase vascular responsiveness. A: mesenteric arterioles isolated and pressurized without flow from SN-salt (○, n = 7) and control (●, n = 8) rats were treated with increasing concentrations of sodium nitroprusside (SNP). B: arterioles from SN-salt rats (n = 8) were treated with increasing concentrations of SNP in the absence (○) and presence (●) of CGRP8–37. Values are expressed as means ± SE.

Fig. 6. RAMP1–3 levels do not change in the vasculature of SN-salt hypertensive and control rats. Protein extracts isolated from SN-salt (n = 6) and control (n = 6) superior mesenteric arteries were fractionated by PAGE and transferred to a nylon membrane. Separate blots were performed for incubation with RAMPs 1–3. Each blot was stripped followed by incubation with the actin antibody. The blot shown is a representative composite blot. RAMP-to-actin ratios were determined by scanning laser densitometry. Values are expressed as means ± SE.
hypotensive response, there were significant differences in Although CGRP and AM both produced a similar global hemodynamics in rats assessed by fluorescent microspheres. AM was found to cause changes in systemic and regional vascular beds to CGRP and AM under normal physiological conditions have been demonstrated (9). In this report, CGRP or AM responsiveness to CGRP, but not AM, would be observed. If CLR was primarily coupled with RAMP1, then an increased scenario, an increase in RCP would enhance CLR activity, and, it is interesting that our isolated vessel results agree with the findings described above; in both cases, the vessels became hyperresponsive to CGRP but not AM. Based on our in vivo studies and observations that CLR and RAMP1–3 did not change appreciably, the lack of a similar increase in AM responsiveness is puzzling. However, these experiments only looked at expression of receptor proteins and not their interactions. It is possible that RAMP2 and -3 may be segregated from CLR under conditions of hypoxia or hypertension, leading to a preferential coupling of CLR with RAMP1. In this scenario, an increase in RCP would enhance CLR activity, and, if CLR was primarily coupled with RAMP1, then an increased responsiveness to CGRP, but not AM, would be observed. Alternatively, differences in the responsiveness of different vascular beds to CGRP and AM under normal physiological conditions have been demonstrated (9). In this report, CGRP or AM was found to cause changes in systemic and regional hemodynamics in rats assessed by fluorescent microspheres. Although CGRP and AM both produced a similar global hypotensive response, there were significant differences in regional vasodilator effects of the two peptides. Indeed, blood flow to the gut was increased by CGRP to a much greater degree than by AM. Our results are consistent with these data, but the mechanism underlying differences in regional organ blood flows evoked by CGRP and AM are not clear.

Our finding that the enhanced vascular responsiveness to CGRP (and AM) was associated with increased levels of RCP is also consistent with other reports demonstrating that RCP is a potential regulator of CLR/RAMP function in vivo. It was previously reported that RCP in dorsal root ganglia and dorsal horn neurons of the rat can be regulated by CGRP receptor blockade and pain-related stimuli (11). From these studies, it was concluded that modulation of RCP expression was directly related to altered signaling capacity at the CGRP receptor. In addition, CGRP also inhibits the contraction of the myometrium during pregnancy and the estrous cycles (12, 18, 20). In the pregnant mouse, there was an increase in myometrial reactivity to CGRP that correlated with an increase in uterine RCP protein. At parturition, there was a rapid decline in RCP content that coincided with the loss of CGRP inhibition of acetylcholine-induced uterine contractions. Similarly, sensitivity of the myometrium to CGRP was found to vary during the mouse estrous cycle in a progesterone-dependent manner, and sensitivity to CGRP correlated with RCP expression. In these studies, CLR and RAMP1 expression was measured but did not correlate with CGRP responsiveness. In this current study, we have demonstrated that the vascular responsiveness to CGRP increased with the increased expression of RCP, similar to that observed previously in the uterus. Interestingly, this enhanced vascular response to CGRP was not accompanied by an increase in either CLR or RAMP1. Thus, it appears that the increased vascular response to CGRP by an upregulation of RCP expression is a novel method of counterregulation for increased blood pressure that results in increased signaling from a constant population of CGRP receptors.

If RCP does play a role in mediating the enhanced vascular reactivity to CGRP (and AM) in SN-salt hypertension, then the question arises as to its function. RCP is a 148-amino acid intracellular protein (4, 14). RCP has no homology to CLR, the RAMPs, or other sequences in GenBank and contains no defined protein motifs that can be used to predict its function (4, 14). Structural analysis of RCP shows that it lacks hydrophobic regions that could serve as transmembrane domains and contains no consensus sequences for lipid attachment, indicating that RCP associates with the membrane through ionic interactions (4, 14). In addition, subcellular localization studies suggest that RCP is a peripheral rather than an integral membrane protein, and it coimmunoprecipitates in a protein complex with CLR and a RAMP protein (4, 14). In vitro analysis of cultured cells that express functional CGRP and AM receptors using an antisense RCP construct for transfection demonstrated that inhibition of RCP expression sharply reduced CGRP- and AM-mediated cAMP production. Loss of RCP did not affect ligand binding to the receptor or receptor density, indicating that RCP did not function as a chaperone, like the RAMP proteins, to route CLR to the cell surface but rather to couple the receptor complex to the cellular signaling pathways. Furthermore, inhibition of RCP expression did not alter signal transduction of other G protein-coupled receptors such as the β2-adrenergic and A2h adenosine receptors (4, 14).
To date, however, very little is known about the regulation of RCP levels in studies where changes in RCP content and CGRP receptor function were examined (11, 12, 18, 20). Likewise, the regulation of CLR and/or RAMP expression is unclear. RAMP1 appears to be induced by dexamethasone treatment of cultured human coronary artery muscle cells, and the cytokine tumor necrosis factor-α decreases CLR and RAMPs 1 and 2 in cultured coronary artery smooth muscle (2a). In addition, there is a complex modulation of CLR and RAMP levels following aortic banding and heart failure (2a). Sex steroids appear to increase CLR and RAMP levels in the rat during pregnancy, and decreased CLR and RAMP1 proteins were observed in vascular tissues in preeclamptic placentas along with decreased CGRP-binding sites (20, 2a).

In summary, these data indicate that the in vivo vascular responsiveness to the dilator activity of CGRP and AM is enhanced in SN-salt hypertension and provide a mechanism to explain how CGRP plays a compensatory depressor role to partially attenuate the blood pressure elevation in the absence of increased neuronal CGRP synthesis and release. Furthermore, this increase in vascular sensitivity may be mediated by an increase in RCP, a protein that represents a novel mechanism for regulating signal transduction at G protein-coupled receptors via enhanced coupling between the ligand-binding component of the receptor and the signaling mechanism(s).

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DISCLOSURES

No conflicts of interest are declared by the authors.

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