Natriuretic Peptide Receptor-C Attenuates Hypertension in Spontaneously Hypertensive Rats
Role of Nitroxidative Stress and Gi Proteins
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Abstract—C-Atrial natriuretic peptide (ANP) 4–23, a ring deleted analog of ANP that specifically interacts with natriuretic peptide receptor-C (NPR-C), has been shown to decrease the enhanced expression of Giα proteins implicated in the pathogenesis of hypertension. In the present study, we investigated whether in vivo treatment of spontaneously hypertensive rats (SHRs) with C-ANP 4–23 could attenuate the development of high blood pressure (BP) and explored the underlying mechanisms responsible for this response. Intraperitoneal injection of C-ANP 4–23 at the concentration of 2 or 10 nmol/kg body weight to prehypertensive SHRs attenuated the development of high BP, and at 8 weeks it was decreased by ≈20 and 50 mm Hg, respectively; however, this treatment did not affect BP in Wistar-Kyoto rats. C-ANP 4–23 treatment of adult SHRs for 2 weeks also attenuated high BP, heart rate, and restored the impaired vasorelaxation toward control levels. In addition, the enhanced levels of superoxide anion (O₂⁻), peroxynitrite, NADPH oxidase activity, and the enhanced expression of Giα proteins, NOX4, p47phox, nitrotyrosine, and decreased levels of endothelial nitric oxide synthase (eNOS or NOS3) and NO in SHRs were attenuated by C-ANP 4–23 treatment; however, the altered levels of NPR-A/NPR-C were not affected by this treatment. In conclusion, these results indicate that NPR-C activation by C-ANP 4–23 attenuates the development of high BP in SHRs through the inhibition of enhanced levels of Giα proteins and nitroxidative stress and not through eNOS/cGMP pathway and suggest that NPR-C ligand may have the potential to be used as therapeutic agent in the treatment of cardiovascular complications including hypertension. (Hypertension. 2014;63:00-00.) • Online Data Supplement

Key Words: atrial natriuretic peptides receptor • blood pressure • nitric oxide synthase type III • oxidative stress • rats, inbred SHR

Natriuretic peptides, including atrial natriuretic peptide (ANP), brain natriuretic peptide, and C-type natriuretic peptide produced in mammalian hearts,1,2 regulate a variety of physiological functions including blood pressure (BP)3 through the interaction and activation of natriuretic peptide receptors (NPRs).

Two major types of NPRs, NPR-A/NPR-B8,9 and NPR-C,7 have been reported. NPR-A and NPR-B are membrane guanyllyl cyclase receptors, whereas NPR-C does not possess guanylyl cyclase activity. Two different subtypes of NPR-C with molecular mass of 67 and 77 kDa have been identified with a broad range of ligands, including ANP, brain natriuretic peptide, C-type natriuretic peptide, and C-ANP 4–23[des(Gln,18 Ser,19 Gly,20 Leu,21 Gly22) ANP4–23-NH2]8; however, C-ANP 4–23 is specific for NPR-C and has no affinity for NPR-A or NPR-B.8,9 The 77-kDa protein is implicated in ligand internalization as a clearance receptor,10 whereas 67-kDa protein is coupled to adenylyl cyclase inhibition through inhibitory guanine nucleotide regulatory protein Gi11 or to activation of phospholipase C (PLC).12 However, we showed that NPR-C activation by C-ANP 4–23 and resultant decrease in cAMP levels contribute to the activation of phosphatidyl inositol turnover signaling and suggested a cross-talk between NPR-C–mediated adenylyl cyclase inhibition and PLC signaling pathways.13

The activity of adenylyl cyclase is regulated by 2 guanine nucleotide regulatory proteins (G proteins): Gs (stimulatory) and Gi (inhibitory).14,15 G proteins are heterotrimeric proteins composed of α-, β-, and γ-subunits. Molecular cloning has revealed 4 different forms of Gsα resulting from the differential splicing of 1 gene and 3 distinct forms of Giα (Giα-1, Giα-2, and Giα-3) encoded by 3 distinct genes.17 All 3 forms of Giα have been shown to be implicated in adenylyl cyclase inhibition.18 Alterations in the levels of Gi proteins and cAMP levels that result in the impaired cellular functions lead to various pathological states including hypertension. An increased expression of Gi proteins and their mRNA in hearts and aortas has been shown in several models of hypertensive rats including spontaneously hypertensive rats (SHRs).3,19–21 Furthermore, the inactivation of Gi proteins by a single injection of pertussis toxin in prehypertensive rats (2-week-old SHRs) has been reported to prevent the development of BP22 suggesting the implication of enhanced expression of Gi proteins in the pathogenesis of hypertension.
C-ANP<sub>4–23</sub> that specifically interacts with NPR-C has been reported to decrease the levels of Gi<sub>α</sub> proteins in A10 vascular smooth muscle cell (VSMC)<sup>23</sup> and in VSMC from SHRs.<sup>24</sup> In addition, C-ANP<sub>4–23</sub> was also shown to attenuate the enhanced oxidative stress in VSMC from SHRs<sup>24</sup> that contributes to the enhanced expression of Gi<sub>α</sub> proteins in SHRs.<sup>25</sup> Taken together, it may be possible that C-ANP<sub>4–23</sub> treatment of SHRs could also attenuate the high BP in SHRs. The present study was undertaken to investigate the effect of in vivo administration of C-ANP<sub>4–23</sub> on the development of high BP in SHRs. We have provided the first evidence that the treatment of prehypertensive SHRs with C-ANP<sub>4–23</sub>, an activator of NPR-C, attenuates the development of high BP in SHRs through the inhibition of enhanced expression of Gi proteins and oxidative stress.

We have provided the first evidence that NPR-C activation by C-ANP<sub>4–23</sub> attenuates high BP through decreasing the enhanced oxidative stress and the augmented levels of Gi<sub>α</sub> proteins. From these results it may be suggested that C-ANP<sub>4–23</sub>, an activator of NPR-C, may be used as a therapeutic agent for the treatment of hypertension.

## Methods

### Materials

C-ANP<sub>4–23</sub> was purchased from Calbiochem. [α-<sup>32</sup>P]ATP was from Amersham. The antibodies against Gi<sub>α</sub> proteins, Nox4, p47<sub>phox</sub>, p22<sub>phox</sub>, and endothelial nitric oxide synthase (eNOS) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals used in these experiments were purchased from Sigma-Aldrich Chemical Co (St Louis, MO).

### Animal Treatment

Male SHRs (2- and 12-week-old) and age-matched Wistar-Kyoto (WKY) rats were purchased from Charles River Canada (St-Constant, Quebec, Canada) and housed at the University of Montreal for 2 days.

Two-week-old rats were injected intraperitoneally with C-ANP<sub>4–23</sub> (2 or 10 nmol/kg body weight [BW]) twice per week for 6 weeks in 0.01 mol/L sodium phosphate buffer, pH 7.0, containing 0.05 mol/L NaCl. Twelve-week-old rats were injected with 10 nmol/kg BW twice per week for 2 weeks. Control WKY and SHRs received vehicle alone. BP (anesthesia-free CODA noninvasive tail-cuff method) and heart rate were monitored twice a week. At the end of the treatment regimen, BP and BW were measured and rats were euthanized by decapitation. The hearts, aortae, and kidneys were dissected out. After taking the heart weight, tissues were frozen immediately in liquid nitrogen and stored at −80°C. Heart:BW ratio was used to determine cardiac hypertrophy. Some aortae were used for cell culture to determine the expression of NPR-C, NPR-A, NADPH oxidase subunits, and eNOS as described previously.<sup>25</sup> All animal procedures used in the present study were approved by the Comité de Déontologie de l’Expérimentation sur les Animaux of the University of Montreal (#99030). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health Publication No.85-23, revised 1996).

### Cell Culture

Aortic VSMCs from 8-week-old SHRs and WKY rats (control group) and C-ANP<sub>4–23</sub>-treated SHRs and WKY rats (after 6 weeks of treatment) were cultured as described in detail in the online-only Data Supplement.

### Preparation of Particulate Fractions

Heart, aorta, and kidney particulate fractions were prepared as described in detail in the online-only Data Supplement.

### Vasorelaxation Measurement

Aortae were dissected out from 14-week-old control and C-ANP<sub>4–23</sub>-treated WKY and SHRs, and vasorelaxation was measured as described in detail in the online-only Data Supplement.

### Blood Pressure and Heart Rate Measurement

BP and heart rate were measured by the CODA noninvasive tail-cuff method and the methodology is described in the online-only Data Supplement. BP was expressed as mmHg and heart rate as bpm.

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**Figure 1.** A and B, Effect of in vivo C-atrial natriuretic peptide (ANP)<sub>4–23</sub> treatment on the development of high blood pressure (BP). Two-week-old spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) rats were injected intraperitoneally with C-ANP<sub>4–23</sub> (2 nmol/kg body weight; A) or (10 nmol/kg body weight; B) or vehicle twice per week for 6 weeks and BP was monitored weekly as described in Materials and Methods. C, D, and E, Effect of in vivo C-ANP<sub>4–23</sub> treatment on high BP, heart rate, and vasorelaxation. Twelve-week-old SHRs and WKY were injected intraperitoneally with C-ANP<sub>4–23</sub> (10 nmol/kg body weight) for 2 weeks and BP (C) and heart rate (D) were monitored twice weekly. Aortae were dissected out after 2 weeks of treatment and used to assess vasorelaxation as described in Materials and Methods. Values are means±SEM of 6 rats in each group. ∗P<0.05, ∗∗P<0.01, and ∗∗∗P<0.001 vs WKY control (CTL); ∗P<0.05, **P<0.01, and ***P<0.001 vs SHR CTL.
**Adenylyl Cyclase Assay**

Adenylyl cyclase activity was determined by measuring \(^{32}\)P-cAMP formation from \([\alpha-^{32}\text{P}]\)ATP, as described in detail in the online-only Data Supplement.

**Western Blotting**

Western blotting of Gi proteins, NPR-A, NPR-C, Nox4, p47\(^{phox}\), nitrotyrosine, and eNOS were performed using specific antibodies as described in detail in the online-only Data Supplement.

**Determination of Superoxide Anion Production and NADPH Oxidase Activity**

Basal superoxide anion production and NADPH oxidase activity in heart, aorta, and kidney were measured using the lucigenin-enhanced chemiluminescence method as described in detail in the online-only Data Supplement.

**Determination of Intracellular NO and Peroxynitrite**

The amounts of intracellular NO and peroxynitrite produced in VSMC were measured using intracellular fluorescent probes, whereas tissue NO was measured using Griess method as described in detail in the online-only Data Supplement.

**Statistical Analysis**

Results are expressed as the mean±SEM. Comparisons between groups were made with 1-way ANOVA followed by Newman–Keuls test or Bonferroni multiple comparison tests. Results were considered significant at a value of \(P<0.05\).

**Results**

**Effect of In Vivo C-ANP\(_{4–23}\) Treatment on the Development of High BP and Heart Rate**

The BP profile is shown in Figure 1A–1C. Mean arterial BP was not significantly different in 3-week-old SHRs compared with age-matched WKY and started to increase from 4 weeks in SHRs. However, intraperitoneal injection of C-ANP\(_{4–23}\) at 2 nmol/kg BW to SHRs decreased the BP by \(\approx 20\) mmHg (Figure 1A), whereas at higher dose of 10 nmol/kg BW, the BP was attenuated by \(\approx 50\) mmHg but not to the control WKY level (Figure 1B). In addition, treatment of 12-week-old SHRs with 10 nmol/kg BW C-ANP\(_{4–23}\) also attenuated the BP and after 2 weeks of treatment the BP was decreased by 60 mmHg (Figure 1C). However, C-ANP\(_{4–23}\) treatment did not affect significantly the BP in WKY rats and did not have adverse effects on the health of animals in the study, because all rats treated with C-ANP\(_{4–23}\) maintained or gained weight during the period of the studies (Table S1 in the online-only Data Supplement). Furthermore, as reported earlier,\(^{26}\) the ratio of heart weight:BW was not different in 8- or 14-week-old SHRs.

![Figure 2](image)

**Figure 2.** Effect of in vivo C-atrial natriuretic peptide (ANP)\(_{4–23}\) treatment on the levels of Gi\(_{-2}\) and Gi\(_{-3}\) proteins in heart and vascular smooth muscle cells (VSMC). VSMC lysates (A and B) and heart membranes (C and D) from 8-week-old spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) rats with or without C-ANP\(_{4–23}\) treatment were subjected to Western blotting using antibodies against Gi\(_{-2}\) (A and C) and Gi\(_{-3}\) (B and D) as described in Materials and Methods. The protein bands were quantified by densitometric scanning. The results are expressed as ratio of Gi protein/Dynein of WKY taken as 100%. Values are mean±SEM of 4 separate experiments. **\(P<0.01\) and ***\(P<0.001\) vs WKY control (CTL), *\(P<0.05\), **\(P<0.01\), and ###\(P<0.001\) vs SHR CTL.
compared with their age-matched WKY rats and was not affected by C-ANP$_{4-23}$ treatment (data not shown). In addition, C-ANP$_{4-23}$ treatment of 12-week-old SHRs also attenuated the increased heart rate by 50% (80 versus 40 bpm; Figure 1D).

**Effect of In Vivo C-ANP$_{4-23}$ Treatment on Vasorelaxation**

To investigate whether the attenuation of BP in SHRs by in vivo treatment with C-ANP$_{4-23}$ is associated with beneficial outcome in vascular reactivity, we examined the dilatory dose response curve of the aorta to carbachol and the results are shown in Figure 1E. The endothelium-dependent vasorelaxation was impaired in aorta from SHRs as compared with WKY rats (maximum relaxation of 45% versus 75%). Treatment with C-ANP$_{4-23}$ restored the impaired relaxation by ≈80%. However, this treatment did not significantly alter the vascular relaxation in WKY rats.

**Effect of In Vivo C-ANP$_{4-23}$ Treatment on G-Protein Levels**

To examine whether C-ANP$_{4-23}$–induced decreased BP is attributed to its ability to decrease the enhanced expression of Gi proteins, the effect of C-ANP$_{4-23}$ treatment on the levels of Gicα-2 and Gicα-3 proteins was examined in hearts and aortic VSMC from control and 8-week-old C-ANP$_{4-23}$–treated SHRs and WKY rats by Western blotting. As shown in Figure 2, the levels of Gicα-2 and Gicα-3 that were significantly enhanced by ≈60% and 50%, respectively, in aortic VSMC (Figure 2A and 2B) and 50% and 40%, respectively, in heart (Figure 2C and 2D) from SHRs compared with WKY rats were significantly reduced by C-ANP$_{4-23}$ treatment. In addition, C-ANP$_{4-23}$ treatment also significantly attenuated the levels of Gicα-2 and Gicα-3 in VSMC but not in heart from WKY rats.

**Effect of In Vivo C-ANP$_{4-23}$ Treatment on G Functions**

To investigate whether the attenuation of enhanced levels of Gicα proteins in VSMC from SHRs by in vivo C-ANP$_{4-23}$ treatment was reflected in Gi functions, the effect of angiotensin II, C-ANP$_{4-23}$, and oxotremorine, which interact with angiotensin II type 1, NPR-C, and muscarinic receptor, respectively, and inhibit adenylyl cyclase activity through Gi proteins, was examined in aortic VSMC from 8-week-old control and C-ANP$_{4-23}$–treated SHRs and WKY rats. Results shown in Figure 3 indicate that angiotensin II–mediated, C-ANP$_{4-23}$–mediated, and oxotremorine-mediated inhibition of adenylyl cyclase in VSMC was significantly greater in SHRs as compared with WKY rats (45%, 48%, and 54% versus 25%, 35%, and 30%), and this enhanced inhibition was significantly attenuated toward control WKY levels by in vivo C-ANP$_{4-23}$ treatment. However, C-ANP$_{4-23}$ treatment did not have significant effect on adenylyl cyclase inhibition in WKY rats.

**Effect of In Vivo C-ANP$_{4-23}$ Treatment on the Expression of NPR-A and NPR-C in SHRs**

The increased levels of NPR-A and decreased levels of NPR-C that have been shown in VSMC from SHRs do not seem to contribute to the increased BP in SHRs because NPR-A knockout mice were shown to exhibit increased BP, whereas NPR-C knockout mice exhibited decreased BP. However, it was of interest to investigate whether C-ANP$_{4-23}$ treatment modulates the levels of NPR-A and NPR-C in SHRs. Results shown in Figure 4 indicate that the levels of NPR-A that were significantly increased by ≈90% in VSMC (Figure 4A) and 75% in heart (Figure 4C) and the levels of NPR-C that were significantly decreased by ≈60% in both VSMC (Figure 4B) and heart (Figure 4D) from SHRs as compared with WKY rats were not altered by C-ANP$_{4-23}$ treatment; however, C-ANP$_{4-23}$ treatment decreased the levels of NPR-C by ≈15% in VSMC but not in heart from WKY rats.

**Effect of In Vivo C-ANP$_{4-23}$ Treatment on the Production of Superoxide Anion and NADPH Oxidase Activity in SHRs**

To investigate whether C-ANP$_{4-23}$–induced attenuation of Gicα protein levels is attributed to its ability to decrease the production of superoxide anion ($O_2^-$), the effect of C-ANP$_{4-23}$ on the $O_2^-$ production was examined in aorta, heart, and kidney from SHRs and age-matched WKY rats. The results shown in Figure 5 indicate that $O_2^-$ was significantly higher by ≈150% in aorta (panel A), 100% in heart (panel B), and 60% in kidney (panel C) from SHRs as compared with WKY. Treatment of SHRs with C-ANP$_{4-23}$ decreased the enhanced $O_2^-$ production by ≈60%, 75%, and 90%, respectively. To further investigate the role of NADPH oxidase in C-ANP$_{4-23}$–induced attenuation of $O_2^-$ production in SHRs, the effect of C-ANP$_{4-23}$ treatment on NADPH oxidase activity was examined in aorta, heart, and kidney from SHRs and WKY rats. The results shown in Figure 6 indicate that NADPH oxidase activity was also significantly enhanced by ≈2-fold in aorta (panel D) and heart (panel E) and 60% in kidney (panel E) from SHRs as compared with WKY and C-ANP$_{4-23}$ treatment completely restored the enhanced activity to control levels. Similar results were also observed in VSMC from SHRs (data not shown).
Effect of In Vivo C-ANP4–23 Treatment on the Expression of NADPH Oxidase Subunits in SHRs

To examine whether C-ANP 4–23–induced decreased NADPH oxidase activity in SHRs is because of its ability to decrease the expression of different subunits of NADPH oxidase, the effect of C-ANP4–23 treatment on the expression of Nox4 and p47phox critical subunits involved in NADPH oxidase activation was examined in VSMC from WKY and SHRs. The results shown in Figure 6 indicate that the levels of Nox4 (panel A) and p47phox (panel B) were significantly enhanced in VSMC from SHRs by ≈40% and 50%, respectively, as compared with WKY rats, and this increase was attenuated to control WKY level by C-ANP4–23 treatment. However, C-ANP4–23 treatment did not alter the levels of Nox4 and p47phox in WKY rats. In addition, the expression of P22phox was not different in VSMC from SHRs and WKY (data not shown).

Effect of In Vivo C-ANP4–23 Treatment on the Levels of eNOS and NO in SHRs

NPR-C activation by C-ANP4–23 has been shown to activate NO synthase in various tissues including smooth muscle33,34 resulting in the increased levels of NO that through cGMP contribute to BP regulation. Because SHRs have been shown to exhibit decreased expression of eNOS,35 it was of interest to examine whether C-ANP4–23–induced decreased BP in SHRs is also attributed to its ability to augment the expression of eNOS. To test this, we determined the effect of in vivo C-ANP4–23 treatment on the levels of eNOS in VSMC, aorta, and kidney from SHRs and WKY rats. Results shown in Figure 7 indicate that the levels of eNOS were significantly decreased by ≈70% in VSMC (panel A) and 50% in aorta (panel B) from SHRs as compared with WKY rats, and C-ANP4–23 treatment further decreased these levels to ≈80%, and 70%, respectively. In addition, the levels of eNOS were also decreased by ≈35% and 20% in VSMC and aorta, respectively, from WKY rats by C-ANP4–23 treatment. However, the levels of eNOS were significantly increased in kidney by ≈20% and this increase was attenuated completely to control levels by C-ANP4–23 treatment (Figure 7C).

To further investigate whether C-ANP4–23–induced decreased/increased expression of eNOS results in decreased/increased levels of NO, we determined the levels of NO in VSMC, aorta, and kidney from control and C-ANP4–23–treated SHRs and WKY rats. Results illustrated in Figure 7D and 7E indicate that the levels of NO were also decreased by 40% in VSMC and aorta from SHRs, and C-ANP4–23 treatment further decreased these levels to 75% and 55%, respectively. In addition, C-ANP4–23 treatment also decreased the levels of NO in VSMC from WKY rats by ≈25%. However, the levels of...
NO were increased by ≈25% in kidney from SHRs and were significantly attenuated by ≈80% by C-ANP4–23 treatment (Figure 7F), whereas this treatment did not have any effect on the levels of NO in WKY rats.

**Effect of In Vivo C-ANP4–23 Treatment on the Levels of Peroxynitrite and Tyrosine Nitration in SHRs**

Because decreased bioavailability of NO has been shown to result in the concomitant increase in the levels of peroxynitrite (ONOO−),36 we determined the levels of ONOO− in VSMC from SHRs compared with WKY rats, and C-ANP4–23 treatment attenuated the levels by ≈60% (130%–55%). However, C-ANP4–23 treatment did not significantly attenuate the ONOO− levels in SHRs which is associated with inhibition of enhanced expression of Giα proteins and nitroxidative stress.

The intraperitoneal injection of C-ANP4–23 into 2-week-old prehypertensive SHRs significantly attenuated the BP in a dose-dependent manner. At a concentration of 2 nmol/kg BW, C-ANP4–23 decreased the BP by ≈20 mm Hg, whereas a decrease of ≈50 mm Hg was achieved at 10 nmol/kg BW of

**Discussion**

Natriuretic peptides, such as C-type natriuretic peptide and ANP that selectively bind to NPR-B and NPR-A, respectively, were shown to decrease BP in 16-week-old SHRs.37,38 However, in the present study, we report for the first time that C-ANP4–23 that is specific for NPR-C and has no affinity for NPR-A or NPR-B3,9 attenuated the development of BP in SHRs which is associated with inhibition of enhanced expression of Gi proteins and nitroxidative stress.

The intraperitoneal injection of C-ANP4–23 into 2-week-old prehypertensive SHRs significantly attenuated the BP in a dose-dependent manner. At a concentration of 2 nmol/kg BW, C-ANP4–23 decreased the BP by ≈20 mm Hg, whereas a decrease of ≈50 mm Hg was achieved at 10 nmol/kg BW of
C-ANP4–23 treatment was also reflected in attenuation of Gi protein expression and NO production in aortic vascular smooth muscle cells (VSMC), heart, and kidney. VSMC lysates, heart, and kidney homogenates from SHRs and WKY rats with or without C-ANP4–23 treatment were subjected to Western blotting using an anti-eNOS antibody as described in Materials and Methods. The protein bands were quantified by densitometric scanning. The results are expressed as ratios of eNOS/Dynein of WKY taken as 100%. Results are expressed as percentages of WKY control group (take as 100%). Values are mean±SEM of 4 separate experiments. *P<0.05, **P<0.01 vs WKY control (CTL); #P<0.05 vs SHR CTL.

C-ANP4–23 treatment decreased the expression of Gα-2 and Giα proteins in VSMC and also the enhanced expression of Giα-3 proteins in VSMC from SHRs as well as in WKY rats. These results are in accordance with our earlier findings showing that C-ANP4–23 treatment decreased the expression of Gα-2 and Giα-3 proteins in A10 VSMC and also the enhanced expression of Giα proteins in VSMC from SHRs. The attenuation of the enhanced expression of Giα proteins by in vivo C-ANP4–23 treatment was not only confined to aortic VSMC but also attenuated the enhanced expression of Giα proteins in hearts as well as in aorta from SHRs (data not shown). The attenuation of the enhanced expression of Giα proteins by in vivo C-ANP4–23 treatment was also reflected in attenuation of Gi functions as demonstrated by decreased inhibition of adenyl cyclase by angiotensin II, C-ANP4–23, and oxotremorine. These results suggest that C-ANP4–23-induced decreased BP in SHRs may be attributed to its ability to attenuate the enhanced expression of Giα proteins that has been shown as a contributing factor in the pathogenesis of hypertension in SHRs. In this regard, the inactivation of Gi proteins by intraperitoneal injection of pertussis toxin into prehypertensive SHRs that results in the decreased expression of Giα proteins has also been shown to attenuate the development of high BP in SHRs. Enhanced oxidative stress has been shown to play a critical role in the pathogenesis of cardiovascular disease including hypertension. Our results showing an enhanced production of O2−, NADPH oxidase activity in heart, aorta, and kidney from SHRs and enhanced expression of NADPH oxidase subunits NOX4 and P47phox in VSMC from SHRs are in agreement with our earlier studies and the studies of other investigators. Furthermore, the fact that in vivo treatment of SHRs with C-ANP4–23 resulted in the attenuation of the enhanced production of O2−, NADPH oxidase activity, and NADPH oxidase subunits NOX4 and P47phox suggests that C-ANP4–23-induced attenuation of high BP may also involve a reduction in oxidative stress. However, the implication of Nox5 that has been shown recently to be expressed in human endocardial cells, human VSMC, and human endothelial cells in enhanced oxidative stress in VSMC from SHRs and its regulation by C-ANP4–23, is not known and needs to be investigated. We earlier showed the implication of enhanced oxidative stress in the enhanced expression of Giα proteins in VSMC from SHR. Thus taken together, it may be suggested that the attenuation of enhanced expression of Giα proteins and resultant decreased BP by C-ANP4–23 treatment may be mediated through the reduction in oxidative stress. However, C-ANP4–23-induced decreased oxidative stress and reduced levels of Giα proteins in heart may not be responsible for the...
attenuation of the development of high BP but may contribute to the restoration of impaired cardiac functions in hypertension. In this regard, allopurinol, an antioxidant and inhibitor of xanthine oxidase, has been shown to significantly improve coronary flow reserve and left ventricular functions in patients with idiopathic dilated cardiomyopathy.43

Several studies have shown a decreased production/bioavailability of NO associated with hypertension,44–46 which may be attributed to the decreased expression of eNOS and to the increased levels of O₂⁻ leading to the maintenance of the elevated peripheral resistance and thereby elevated BP.47 In the present study, we demonstrate that the VSMC and aorta from SHRs exhibit decreased levels of NO which may be because of the decreased expression of eNOS as well as to the enhanced levels of O₂⁻. In this regard, the decreased expression of eNOS and NO has been reported in cardiac myocyte and vascular smooth muscle of hypertensive animals35,48,49 and suggests that the increased BP in SHRs may also be attributed to the decreased levels of NO. Furthermore, eNOS knockout mice were shown to exhibit increased BP.50 In addition, the inhibition of NO synthase by L-NAME (N⁵-nitroarginine methyl ester) has also been reported to augment the BP and the expression of Giri proteins in rats.51 However, our results are in contrast with the study of Caniffi et al37 who have reported an increased expression of eNOS in heart and aorta from SHRs. This apparent discrepancy may be because of the age difference of SHRs used in their study (16 versus 8 weeks). However, we showed that kidney from SHRs exhibits an enhanced expression of eNOS and enhanced levels of NO. Our results are in accordance with the studies of other investigators who have demonstrated an enhanced expression of iNOS (inducible nitric oxide synthase) and eNOS and an elevation of renal NOS activity in 12-week-old SHRs52 and 20-week-old SHRs compared with WKY.53 In addition, Elesgaray et al38 have shown the overexpression of all the 3 isoforms, eNOS, iNOS, and nNOS (neuronal nitric oxide synthase), in kidney from SHRs compared with WKY rats. Thus taken together, it may be suggested that the upregulation of NOS isoforms in kidney may not contribute to the development of high BP and may play a compensatory role in response to an increased BP in SHRs.

Several studies have shown the interaction of natriuretic peptides and NO in the regulation of BP.37,38,44,45 In addition, NPR-C activation by C-ANP has also been reported to activate eNOS in various tissues including smooth muscle.33 We also

Figure 8. Effect of in vivo C-atrial natriuretic peptide (ANP) treatment on ONOO⁻ production and the levels of nitrotyrosine protein in vascular smooth muscle cells (VSMCs), aorta, and kidney. A, Confluent VSMCs were incubated at 37°C for 1 hour with fluorescent probes and peroxynitrite levels were measured as described in Materials and Methods. Results are expressed as percentages of Wistar-Kyoto (WKY) control group (taken as 100%). Values are mean±SEM of 4 separate experiments. VSMC lysates (B), aorta (C), or kidney (D) homogenates from spontaneously hypertensive rats (SHRs) and WKY rats with or without C-ANP treatment were subjected to Western blotting using anti-nitrotyrosine antibody as described in Materials and Methods. The protein bands were quantified by densitometric scanning. The results are expressed as ratio of nitrotyrosine/β-actin of WKY taken as 100%. Values are mean±SEM of 4 separate experiments. *P<0.05, **P<0.01, and ***P<0.001 vs WKY control (CTL); ##P<0.01, ### P<0.001 vs SHR CTL.
examined whether C-ANP4–23–induced attenuation of BP in SHRs is attributed to its ability to augment the levels of eNOS and NO; however, in the present in vivo study, we demonstrate that C-ANP4–23 treatment decreased the enhanced levels of NO and eNOS in kidney and attenuated further the decreased levels of eNOS and NO in VSMC and aorta from SHRs and suggest that C-ANP4–23–induced decreased BP may not involve NO and NO-stimulated cGMP pathway. Furthermore, our results showing that the levels of ONOO• and nitrotyrosine were increased in VSMC, aorta, and kidney from SHRs that may be formed by the reaction of NO with O2− are in accordance with the studies of other investigators who have also reported an increased levels of ONOO• and nitrotyrosine content in aorta and mesenteric arteries as well as in kidney from SHRs without and with typical symptoms of metabolic syndrome. However, we report for the first time that in vivo C-ANP4–23 treatment decreased the enhanced levels of ONOO• and nitrotyrosine in VSMC, aorta, and kidney from SHRs. Thus, it may be suggested that C-ANP4–23–induced decreased levels of NO because of decreased expression of eNOS, O2−, and ONOO• and resultant decreased nitrosative stress may be responsible for C-ANP4–23–induced attenuation of high BP. In this regard, C-ANP4–23 has been reported recently to decrease nitrosative stress induced by ammonia in astrocytes. Furthermore, the fact that the decreased levels of NPR-C and increased levels of NPR-A in VSMC from SHRs were not affected by C-ANP4–23 treatment suggests that these receptors may not be implicated in the attenuation of BP induced by C-ANP4–23.

Perspectives

Hypertension is a multifactorial disease. The present study demonstrates an important role of NPR-C in the regulation of BP. Moreover, it shows for the first time that NPR-C activation by C-ANP4–23, a specific agonist, decreases the BP in SHRs by inhibiting the enhanced expression of Gαi proteins and nitrooxidative stress, the contributing factors for the pathogenesis of hypertension without altering the levels of NPR-C or NPR-A proteins. Based on these findings, it can be suggested that NPR-C agonists, C-ANP4–23, or others may have the potential to be used as therapeutic agents in the treatment of cardiovascular complications including hypertension in animals/individuals who are genetically predisposed to hypertension. The advantages of C-ANP4–23–like therapeutics would be widespread in light of the World Health Organization’s prediction that 51% of the total number of work hours expected to be lost because of noncommunicable diseases from 2011 to 2025 will be solely because of cardiovascular diseases.

Acknowledgments

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Disclosures

None.


Natriuretic Peptide Receptor-C Attenuates Hypertension in Spontaneously Hypertensive Rats: Role of Nitrooxidative Stress and Gi Proteins
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Natriuretic peptide receptor C attenuates hypertension in Spontaneously Hypertensive Rats: Role of nitrooxidative stress and Gi proteins*

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Detailed Methods

Cell culture

Aortic VSMC from 8-week-old SHR and their age-matched WKY rats (control group) and C-ANP4-23-treated SHR and WKY rats (after 6 weeks of treatment) were cultured as described previously. The purity of the cells was checked by immunofluorescence technique using α-actin as described previously. These cells were found to contain high levels of smooth muscle-specific actin. The cells were plated in 75 cm² flasks and incubated at 37°C in 95% air and 5% CO₂ humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM) (with glucose, L-glutamine, and sodium bicarbonate) containing antibiotics and 10% heat-inactivated fetal bovine serum (FBS). Confluent cell cultures were starved by incubation for 3h in DMEM without FBS at 37°C to reduce the interference by growth factors present in the serum. Cell lysates were prepared and used for Western blotting and adenylyl cyclase activity determination.

Preparation of Particulate Fractions

Heart, aorta and kidney particulate fractions were prepared as described previously. The dissected organs were quickly frozen in liquid nitrogen, pulverized to a fine powder using a mortar and pestle cooled in liquid nitrogen and stored at −80°C until assayed. The powder was homogenized (12 stokes) in a teflon glass homogenizer, in a buffer containing 10 mM Tris-HCl, 1 mM EDTA (pH 7.5). The homogenate was centrifuged at 1,000 g for 10min. The supernatant was discarded, and the pellet was finally suspended in the above buffer and used for Western blotting.

Adenylyl Cyclase Assay

Adenylyl cyclase activity was determined by measuring \[^{32}\text{P}]\text{cAMP} \text{formation from} \[^{\alpha-32}\text{P}]\text{ATP, as described previously} \text{. Briefly, the assay medium containing} \text{50 mM glycylglycine, pH 7.5, 0.5 mM MgATP,} \[^{\alpha-32}\text{P}]\text{ATP (1.5–10) ×10}^6 \text{c.p.m.), 5 mM MgCl}_2 \text{(in excess of the ATP concentration), 100 mM NaCl, 0.5 mM cAMP, 1 mM IBMX, 0.1 µM EGTA, 10 µM GTP}^\gamma_S \text{and an ATP-regenerating system consisting 2 mM phosphocreatine, 0.1 mg of creatine kinase/ml and 0.1 mg of myokinase/ml in a final volume of 200 µl was preincubated for 2 min at 37 °C. The reaction was initiated by the addition of the membrane proteins (20-30 µg) to the reaction mixture. The reactions were terminated after 10 minutes by the addition of 0.6 ml of 120 mM zinc acetate. cAMP was purified by co-precipitation of other nucleotides with ZnCO}_3 \text{, by addition of 0.5 ml of 144 mM Na}_2\text{CO}_3 \text{and subsequent chromatography by the double-column system, as described by Salomon et al} \text{. To examine the receptor-dependent functions of Gi proteins, adenylyl cyclase activity was determined in the presence of 10µM GTP}^\gamma_S \text{alone, or in combination with angiotensin II (10}^{-5}\text{M), C-ANP}_{4-23} \text{ (10}^{-7}\text{M) or oxotremorine (50µM) as described previously} \text{.}

Western blotting

Western blotting of Gi proteins, NPR-A, NPR-C, Nox4, p47^{phox} and p22^{phox} (NADPH oxidase subunits), nitrotyrosine and eNOS were performed using specific antibodies as described previously. After SDS-PAGE, the separated proteins were electrophoretically transferred to nitrocellulose paper (Schleicher and Schuell Inc., Keene, N.H.) with a semi-dry transblot apparatus (Bio-Rad Laboratories, Mississauga, Ontario) at 15 V for 45 min. After transfer, the membranes were washed twice in
phosphate-buffered saline (PBS) and were incubated in PBS containing 8% dehydrated milk at room temperature for 2h. The blots were then incubated with respective primary antibodies; L-5 for Giα-2, C-10 for Giα-3, N-15 for Nox4, D-10 for p47phox and C-17 for p22phox antibodies in PBS containing 3% dehydrated milk and 0.1% Tween-20 at room temperature for 2h. The antibody-antigen complexes were detected by second antibody at room temperature for 1h and protein bands were visualized by enhanced-chemioluminescence. Quantitative analysis of specific bands was performed by densitometric scanning of the autoradiographs with an enhanced laser densitometer (LKB Ultroscan XL, Pharmacia, Dorval, Que.) and quantified using gelscan XL evaluation software (version 2.1) from Pharmacia.

**Determination of Superoxide anion production and NADPH oxidase activity**

Basal superoxide anion production and NADPH oxidase activity in heart, aorta and kidney was measured using the lucigenin-enhanced chemiluminescence method with low concentration (5µmol/l) of lucigenin as described previously. The heart, aorta and kidney tissues from control and C-ANP4-23-treated SHR and WKY rats were washed in oxygenated Kreb–Hepes buffer, and placed in scintillation vials containing lucigenin solution, and the emitted luminescence was measured with a liquid scintillation counter (Wallac 1409; Perkin Elmer Life Science, St Laurent, Quebec, Canada) for 5 min. The average luminescence value was estimated, the background value subtracted and the result was divided by the total weight of tissue in each sample.

The NADPH oxidase activity in the samples was assessed by adding 10⁻⁴ mol/l NADH (Sigma Chemical Co.) in the vials before counting. Basal superoxide- induced luminescence was then subtracted from the luminescence value induced by NADH.

**Determination of Intracellular NO and Peroxynitrite**

The amounts of intracellular NO, and peroxynitrite produced in VSMC were measured using intracellular fluorescent probes, diaminofluorescein-2 diacetate (DAF-2DA) and dihydrorhodamine 123 respectively as described earlier. Confluent VSMC, after washing twice with phosphate buffer solution (PBS) were incubated at 37°C for 1 hour with both 10 mmol/L DAF-2 DA and 10⁻⁶ mol/L acetylcholine for detecting NO, and with 5x10⁻⁵ mol/L DHR for detecting peroxynitrite, respectively. Cells were washed twice with PBS, and fluorescence intensities were measured by a spectrophotometer (TECAN infinite 200 PRO) with excitation and emission wavelengths at 495 nm and 515 nm for DAF-2DA, and 480 nm and 530 nm for DHR, respectively. Changes in fluorescence intensities were expressed as percentages of the values obtained in the WKY control group (taken as 100%).

The amount of NO in aorta and kidney was measured using Griess method. Organs were dissected out and homogenized in bicarbonate buffer (0.2M, pH 9.2) in 1:10 w/v ratio. To 100 µl of homogenate, 50µl of Griess reagent (Sigma Chemical Co.) was added and the volume made up to 500 µl with water. The mixture was incubated at room temperature for 10 min and absorbance was measured at 530nm. Sodium nitrite was used to generate a standard curve. Values were calculated as µmol NO/g tissue and expressed as percentages of the values obtained in the WKY control group (taken as 100%).

**Vascular reactivity**
Aortae were dissected out from 14-week-old control and C-ANP4-23-treated WKY and SHR and cleaned of fat and connective tissue. Aortae were placed in cold Krebs buffer solution (118 M NaCl, 4.65 M KCl, 25 M NaHCO3, 2.5 M CaCl2, 1.18 M MgSO4, 1.18 M KH2PO4, and 5.5 M dextrose, pH 7.4) and vascular reactivity was measured as per a previously standardized protocol 13. Briefly, aortae were cut into consecutive rings of 2-3mm and submerged in individually jacketed organ baths (RadnotiGlass, Monrovia, CA) pre-warmed to 37°C. The buffer in the baths was oxygenated using a mixture of 95% O2 and 5% CO2. The rings were gently rubbed with an 18-gauge needle to remove the endothelium which was equilibrated for 1 hour under passive tension. Repeated washing and tension adjustments were performed to determine the optimal tension (2 g) for all four groups of rats. Following equilibration 1.0 µM phenylephrine was added to the buffer till a plateau response was achieved. At this point carbachol (0.1mM) was added to complete removal of endothelium. Tension was measured using force-displacement transducers and recorded on a computerized data acquisition system running on WorkBench software (Kent Scientific, Litchfield, CT). Vascular reactivity was expressed as percentage of relaxation of WKY CTL.

Blood Pressure and Heart Rate Measurement

BP and heart rate were measured by the CODA™ non-invasive tail cuff method as previously described14. The CODA™ tail-cuff BP system uses Volume Pressure Recording (VPR) sensor technology to measure the tail BP. VPR recording is clinically validated and provides 99% correlation with telemetry and direct BP measurements. BP was expressed as mmHg and heart rate as beats/min.

References


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**Table S1: Body weight of rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>BW at 8 weeks (g)</th>
<th>BW at 14 weeks (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY CTL</td>
<td>162±5.8</td>
<td>269±6.7</td>
</tr>
<tr>
<td>WKY C-ANP$_{4.23}$</td>
<td>164±6.1</td>
<td>283±9.6</td>
</tr>
<tr>
<td>SHR CTL</td>
<td>153±8.1</td>
<td>271±10.9</td>
</tr>
<tr>
<td>SHR C-ANP$_{4.23}$</td>
<td>151±5.3</td>
<td>276±6.7</td>
</tr>
</tbody>
</table>