Immediate and Sustained Blood Pressure Lowering by Urocortin 2
A Novel Approach to Antihypertensive Therapy?

Thomas Dieterle, Silvia Meili-Butz, Katrin Bühler, Christian Morandi, Dietlinde John, Peter T. Buser, Jean Rivier, Wylie W. Vale, Kirk L. Peterson, Marijke Brink

Abstract—Recently, novel corticotropin-releasing factor-related peptides, named urocortin 1, 2, and 3, and a distinct cardiac and peripheral vascular receptor (corticotropin-releasing factor receptor 2) were described being part of a peripheral corticotropin-releasing factor system modulating cardiovascular function in response to stress. Vasorelaxation and blood pressure lowering have been reported after acute administration of these peptides. No data are available on the acute and chronic effects of urocortin 2 on blood pressure in models of arterial hypertension. To test these effects, hypertensive salt-sensitive and normotensive salt-resistant Dahl rats were randomly assigned to twice-daily applications of urocortin 2 or vehicle for 5 weeks. Blood pressure, heart rate, and left ventricular dimension and function were recorded at baseline, after initial application, and, together with cardiac and aortic expression of urocortin 2 and its receptor, after 5 weeks of treatment. Urocortin 2 significantly reduced blood pressure in hypertensive rats without affecting heart rate. Long-term urocortin 2 treatment in hypertensive rats induced sustained blood pressure reduction and diminished the development of hypertension-induced left ventricular hypertrophy and the deterioration of left ventricular contractile function. Corticotropin-releasing factor receptor 2 expression was preserved despite chronic stimulation by urocortin 2. In conclusion, our study shows that, in an animal model of arterial hypertension, urocortin 2 has immediate and sustained blood pressure-lowering effects. Beneficial effects on blood pressure, left ventricular dimension, and function, together with preserved receptor expression, suggest that corticotropin-releasing factor receptor 2 stimulation by urocortin 2 may represent a novel approach to the treatment of arterial hypertension. (Hypertension. 2009;53:739-744.)

Key Words: CRF receptor ■ urocortin 2 ■ Dahl salt-sensitive rat ■ arterial hypertension ■ blood pressure ■ left ventricular hypertrophy ■ left ventricular function

Arterial hypertension remains the major risk factor for cardiovascular and related diseases. In its most recent report, the World Health Organization lists high blood pressure (BP) as the leading cause of death worldwide. One of the critical consequences of arterial hypertension is structural remodeling of the heart, referred to as left ventricular (LV) hypertrophy (LVH). This response represents the anatomic precursor of a spectrum of maladaptations that are collectively referred to as hypertensive heart disease. Other than being a sequel of arterial hypertension, LVH is the most potent predictor of adverse cardiovascular outcomes in the hypertensive population and an independent risk factor for coronary heart disease, sudden death, heart failure, and stroke. Prevention of LVH and the progression to overt cardiovascular disease are therefore major goals of antihypertensive therapy. Therapeutic efficacy of currently available antihypertensive drugs, however, may be limited by poor compliance, adverse effects, and a poor response to treatment. Thus, there remains a continuous need for novel therapeutic approaches.

In recent years, novel corticotropin releasing factor (CRF)-related peptides and a specific CRF receptor system residing in the heart and peripheral vessels have been identified. Originally identified as a transmitter involved in the regulation of the hypothalamic-hypopituitary-adrenal axis of the stress response, CRF was the first endogenous ligand of the CRF family of peptides to bind to CRF receptors. Three additional CRF-related peptides, named urocortin (Ucn) 1, 2 (human ortholog: stresscopin-related peptide10,11), and Ucn3 (human ortholog: stresscopin14,12), signaling through 2 G protein–coupled receptors, the CRF receptors (CRFR) 1 and 2, have been discovered. Although CRFR1 and CRFR2
are found in the central nervous system, CRFR2 is particularly abundant in the periphery, including the heart and systemic vasculature. Ucn2 and Ucn3 bind selectively to CRFR2, with no appreciable activity at CRFR1.10,12

Marked inotropic and lusitropic effects, as well as a reduction of peripheral resistance, have been reported for Ucn2 in a recent study in wild-type mice and cardiomyopathic mice with congestive heart failure (CHF)18 and, together with beneficial renal and endocrine effects, in pacing-induced CHF in sheep.19 Potent vasodilatory effects by Ucn2 were reported in rat thoracic aorta20 and in human arteries.21 Intravenous application of Ucn2 induced a dose-dependent rapid and highly significant BP reduction in normotensive rats.22–24

To date, BP lowering effects of Ucn2 have only been tested acutely and in normotensive animals but never in animal models of arterial hypertension. No data are available on the long-term effects of Ucn2 treatment on BP, heart rate (HR), cardiac dimension, and function. These data are needed to judge the potential clinical applicability of Ucn2 for the treatment of high BP. We performed a long-term study in the Dahl salt-sensitive (DSS) rat, an animal model of arterial hypertension and LVH. To determine acute and long-term effects, BP measurements, together with echocardiographic analysis of LV dimensions and function, were performed after initial Ucn2 application and at prespecified time points during the chronic treatment phase.

Materials and Methods

Experimental Animals

Male DSS rats (SS/JrHsd, n = 20) and Dahl salt-resistant rats (DSR; SR/JrHsd, n = 20), aged 4 weeks, were obtained from Harlan Inc. Animals were maintained at 20±2°C and 55±20% humidity, with 12/12-hour light/dark cycles and free access to food and water. Animal protocols were approved by the Veterinary Department of Basel and conformed to the rules of the Swiss Federal Act on Animal Protection 1998 and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Treatment Protocol

To induce arterial hypertension, rats were fed a low-salt diet (0.3% NaCl) and, at the age of 6 weeks, were switched to a high-salt diet (HSD) containing 4% NaCl. Age-matched DSR rats were treated in an identical manner and served as normotensive controls. After 15 days of HSD (age 8 weeks), DSS and DSR rats were randomized to a treatment with human Ucn2 at a dose of 2.5 μmol/kg BW/d or the corresponding volume of vehicle (0.9% NaCl). Human Ucn2 was chosen to ensure comparability with previous studies experimental studies.22,24

Ucn2 or vehicle was administered IP in strict 12-hour intervals. Ucn2 was provided by Dr Jean Rivier (Salk Institute).

Echocardiography and BP Measurements

BP measurements and echocardiographic analysis of LV dimensions and function were performed simultaneously on slightly sedated (pentobarbital 20 mg/kg of BW IP), spontaneously breathing animals. Great care was taken to perform the application of Ucn2 or vehicle, BP measurements, and echocardiographic tracings at equal time points after application of pentobarbital. BP was measured at baseline (before the first injection of Ucn2 or vehicle [BL]) and at 5, 10, 15, and 30 minutes after Ucn2 or vehicle injection. Echocardiography was performed in parallel at BL and at 15 and 30 minutes after the first injection of Ucn2 or vehicle, as well as at weeks 1, 2, and 5 of BID treatment, in which case the measurement was always taken at 12 hours after the last application of Ucn2.

Systolic BP was assessed using a standard tail-cuff BP monitor (IITC Inc. Life Science Instruments). Echocardiography was performed using a 15.0-MHz linear transducer interfaced with a Philips SONOS 5500 system (Philips Medical Systems).

For image acquisition, rats were placed in the left lateral decubitus position. The transducer was placed on the left hemithorax. Care was taken not to apply excessive pressure on the chest to avoid bradycardia. The 2D parasternal short-axis view was used as a guide, and an LV M-mode tracing was obtained close to the papillary muscle level with a sweep speed of 150 mm/s. Pulsed Doppler tracings of LV outflow tract velocity were obtained in a modified parasternal long-axis view at a sweep speed of 150 mm/s. Transmitral Doppler flows (E and A velocities) were measured in a modified apical 4-chamber orientation with the sample volume placed at the tips of the mitral leaflet. M-mode and Doppler tracings were recorded on a magneto-optical disk for offline analysis.

LV end-diastolic and end-systolic internal diameters (LVEDD and LVESD), as well as LV posterior wall thickness (PWall), were measured in 3 consecutive heart cycles using the American Society of Echocardiography leading-edge method23 by an investigator blinded for treatment allocation and phase. LV fractional shortening (FS) was calculated as FS (%)=[(LVEDD−LVESD)/LVEDD]×100. Using the mean aortic ejection time (ET) from 3 consecutive heart cycles obtained from the Doppler tracings of the LV outflow tract, we calculated the velocity of circumferential fiber shortening (Vcf) as Vcf (circumferences/s)=(πLVEDD−πLVESD)/(ET×πLVEDD). Mean Vcf provides an in vivo assessment of myocardial contractility under basal conditions and in the absence of acute changes in arterial pressure.24 From E and A velocities, the E/A ratio was calculated as a measure for LV diastolic filling properties.

Tissue Harvesting and Processing

After the last BP measurement and echocardiography, rats were euthanized using thiopental anesthesia (150 mg/kg BW IP). After thoracotomy, hearts were immediately isolated and further processed in ice-cold saline solution. Wet heart weight (HW) and BW were determined, and for each animal the HW/BW ratio was calculated. The LV was separated and rapidly frozen in liquid nitrogen.

RNA Isolation and Analysis by Real-Time PCR

Expression levels of Ucn2, CRFR2, and B-type natriuretic peptide (BNP) in LV and aortic tissue from DSS and DSR rats (DSS; n=7; DSR; n=6) were determined using real-time PCR. Expression of BNP served as a marker of cardiac hypertrophy.27 Total RNA was extracted using TRI-Reagent (Sigma), treated with DNase (RNase-free, Ambion) to remove DNA contaminations, and 2 μg of this RNA were reverse transcribed by random priming (OmniScript reverse transcription kit, Qiagen). Nontranscribed RNA served as negative control. The resulting cDNA was analyzed either undiluted for Ucn2 or diluted 10- and 20-fold for CRFR2 and BNP, respectively. Real-time PCR was performed in an ABI 7500 Fast Sequence Detection System (Applied Biosystems) using iTaq SYBR Green PCR Master Mix (Bio-Rad) and the primers given in Table S1 of the data supplement (available online at http://hyper.ahajournals.org). Primers were designed as intron spanning except for Ucn2, because its gene consists of 1 exon only. β-Tubulin mRNA was used as an internal standard for sample normalization. β-Tubulin by itself did not differ between the experimental groups (Table S2). mRNA levels of the target and standard genes were calculated by using a standard curve. All of the samples were assayed in triplicate.

Statistical Analysis

Statistical analysis was performed using GB-STAT software (Dynamic Microsystems Inc, version 8.0). All of the values are expressed as means±SDs. Comparisons of HW/BW ratios and expression levels of Ucn2 and CRFR2 at week 5 of treatment with Ucn2 or...
vehicle were performed using ANOVA. Changes in BP, LV dimension, and LV function during the treatment were analyzed using repeated-measures ANOVA. Student-Newman-Keuls test was used for posthoc comparisons. \( P<0.05 \) was considered to be statistically significant.

**Results**

**BP Is Lowered and HR Remains Unchanged After Ucn2 Treatment**

Starting at the age of 6 weeks, DSS and DSR rats were fed an HSD containing 4% NaCl. At this age, systolic BP was 114±17 mm Hg and 106±11 mm Hg in DSS and DSR rats, respectively (\( P \) value not significant). Systolic BP measured after 2 weeks of HSD was significantly higher in DSS rats compared with time- and diet-matched DSR rats (153±7 mm Hg versus 120±3 mm Hg; \( P<0.05 \)). At this time point, Ucn2 treatment was started. BL BP did not differ between the animals assigned to treatment with Ucn2 and those assigned to the vehicle-treated group. The changes over time in systolic BP after acute Ucn2 administration (2.5 \( \mu \)g/kg IP) or during chronic treatment (2.5 \( \mu \)g/kg IP BID) are shown in Figure 1A. Whereas BP continued to rise in vehicle-treated DSS rats, we found sustained BP-lowering effects in animals treated with Ucn2 at a dose of 2.5 \( \mu \)g/kg BID (Figure 1B). Interestingly, no acute or chronic effects on BP were observed in Ucn2-treated DSR rats compared with vehicle-treated animals (Figure 1C and 1D), HR did not differ between groups at any phase of the study. Importantly, the drop in BP in Ucn2-treated DSS rats was not accompanied by a rise in HR (Figure S1).

**Changes in LV Dimension and Function After Ucn2 Treatment**

Effects of Ucn2 on LV dimension and function in hypertensive DSS rats are summarized in Figure 2. Initial application of Ucn2 induced an immediate improvement of FS and Vcf, a measure of contractile force in the basal state. This was accompanied by a reduction of LVEDD and an increase in PWth. Application of vehicle to DSS rats had no effects. E/A ratio at BL did not differ between DSS and DSR rats and was not affected by initial application of Ucn2 or vehicle. Moreover, Ucn2 or vehicle did not change contractile force in normotensive DSR rats (data not shown).

Analysis of LV function after 5 weeks of BID treatment revealed preserved FS in both Ucn2- and vehicle-treated DSS rats. However, contractile force expressed as Vcf was significantly decreased in vehicle-treated DSS rats and maintained in Ucn2-treated DSS rats (\( P<0.05 \)) compared with BL. Importantly, E/A ratios remained unchanged in Ucn2-treated DSS rats after 5 weeks of BID treatment (1.69±0.15 [BL] versus 1.61±0.09 [week 5]; \( P \) value not significant), whereas we observed a significant increase in vehicle-treated DSS rats compared with BL (1.61±0.09 [BL] versus 1.95±0.29 [week 5]; \( P<0.05 \)). No changes were observed in DSR rats treated with Ucn2 or vehicle for 5 weeks.

Figure 2 shows that, after 5 weeks of treatment and measured at the trough point after Ucn2 injection, no difference in LVEDD existed, but importantly, PWth was significantly lower in Ucn2-treated DSS rats than in vehicle-treated controls (0.143±0.010 cm versus 0.191±0.011 cm; \( P<0.05 \)). Analysis of HW/BW ratios (Figure 3A) or HW/tibial length ratios (data not shown) after sacrifice of the DSS rats confirmed the echocardiographic findings. Although HW/BW ratios were significantly higher in DSS compared with DSR rats, among the DSS rats, the Ucn2-treated group displayed a 14% lower HW/BW ratio than the vehicle-treated group.
group (35±3 mg/g versus 40±5 mg/g; \( P<0.05 \)). No differences in HW/BW between treatment groups were observed in DSR rats (28±2 mg/g versus 28±1 mg/g; \( P \) value not significant). Assessment of BNP expression by real-time PCR (Figure 3B) as a marker of hypertrophy did not, however, reveal any differences between the treatment groups. Taken together, our echocardiographic and cardiac weight data show that hearts of Ucn2-treated DSS rats were less hypertrophied than those of vehicle-treated rats after 5 weeks of HSD.

**Gene Expression of CRFR2 and Ucn2**

To evaluate whether Ucn2 treatment changes tissue expression of its receptor, CRFR2, and of endogenous Ucn2, we quantified in LV and aorta the mRNAs that encode these proteins. Neither in DSS nor in DSR rats did LV or aortic CRFR2 mRNA levels differ between the Ucn2 and vehicle groups after 5 weeks of treatment (Figure S2). For Ucn2 mRNA, no statistical significance was obtained in either tissue between the treatment groups. Ucn2 mRNA was present only at very low levels in both tissues, with consequently high variability of our measurements.

**Discussion**

In this study, we investigated the effects of Ucn2, a selective CRFR2 agonist, on BP and LV geometry and function in a clinically relevant and widely used animal model of arterial hypertension and LVH: the DSS rat. The DSS rat demonstrates many features observed in human hypertension, such as an overactivity of the renin-angiotensin system,\textsuperscript{28} as well as an overactivity of the sympathetic nervous system.\textsuperscript{29} In addition, DSS rats have been used to test the effects of drugs from virtually all classes of antihypertensive drugs, thus representing an ideal animal model to test potential new antihypertensive agents.

**Effects of Ucn2 on BP**

Our study is the first to demonstrate immediate and prolonged BP-lowering effects of Ucn2 in arterial hypertension. In humans, a plasma half life of Ucn2 of 15.5 minutes has been reported.\textsuperscript{30} In our study, significant BP reductions were still measured at 12 hours after Ucn2 application, indicating long-lasting effects of Ucn2. Earlier studies have reported vasodilatory and hypotensive effects of Ucn2 in normoten-
sive rats.22,24–26 Our experiments confirm these previous findings and extend them to a clinically relevant model of arterial hypertension: the DSS rat. We demonstrate an immediate and highly significant BP reduction after application of a single dose of Ucn2 with BP values comparable to those observed in normotensive control animals. Despite the rapid drop of BP, no significant increase in HR was observed, a finding of potential clinical importance.

No downregulation of CRFR2 mRNA expression was detected in LV and aortic tissue, which explains, at least in part, the preserved reactivity to CRFR2 stimulation with Ucn2 in DSS rats even after 5 weeks of treatment. Moreover, our findings further support the notion of an involvement of the CRF-related peptides and their receptors in the regulation of cardiovascular function.31

An additional, clinically important observation in this study was that BID application of Ucn2 for 5 weeks led to sustained BP reduction in hypertensive DSS rats, whereas a continuous further increase of BP was found in vehicle-treated animals. These data provide first evidence of a potential beneficial long-term effect of CRFR2 stimulation on BP.

Several mechanisms seem to be involved in the BP-lowering effects of Ucn2. Both endothelium-dependent and -independent vasorelaxation have been described previously in the human internal mammary artery32 and the coronary circulation of pigs.33 The endothelium-dependent component appears to act primarily via the release of endothelial NO, which, in turn, stimulates Ca2+-activated K+ channels in vascular smooth muscle via cGMP-dependent mechanisms.32,34 Moreover, vasodilatory effects via p38 mitogen-activated protein kinase and the protein kinase A pathway may contribute to the vasodilatory effect of Ucn2.22 In addition, potent diuretic effects of Ucn2 have been described at least in an animal model of CHF35 that might contribute to the BP-lowering effects of Ucn2 beyond direct vascular effects.

However, some potential limitations have to be acknowledged with respect to the effects of Ucn2 on BP in this study. To ensure comparability with previous studies in normal rats,22,24 this study was conducted using the human form of Ucn2. Although the rat and human forms of Ucn2 share an 83% homology,36 it is conceivable that the amino acid differences might result in greater or lesser activation of rat CRF2 by the human compared with the rat form of Ucn2. Therefore, the observed effects of Ucn2 might have been more or less potent had the rat form of Ucn2 been used.

Effects of Ucn2 on LV Structure

The goal of antihypertensive therapy is to prevent the development of hypertensive target organ damage and cardiovascular disease, in particular, LVH and CHF. In previous studies, mitogenic effects of Ucn1 have been described in cardiac myocytes and nonmyocytes,37,38 and exposure of cultured cardiac myocytes to Ucn2 and Ucn3 resulted in increased myocyte size and protein synthesis.39 All 3 CRF-related peptides increased markers of hypertrophy in these experiments, with Ucn3 being the most and Ucn2 the least potent. In our study we did not find any differences between Ucn2- and vehicle-treated normotensive DSR rats regarding HW/BW ratio, BNP mRNA expression levels, or echocardiographic LV dimensions. Thus, chronic administration of Ucn2 does not seem to induce relevant hypertrophic effects in the myocardium in normotensive rats. In fact, in hypertensive DSS rats, chronic HW/BW ratio was significantly lower in Ucn2- compared with vehicle-treated animals. Together with unchanged echocardiographic LV diameters and reduced LVFPth, data from this study suggest that chronic administration of Ucn2 may even diminish the hypertension-induced LV hypertrophic response.

Effects of Ucn2 on LV Function

Several recent studies with animal models of dilated cardiomyopathy or pacing-induced CHF have demonstrated potent beneficial effects of Ucn2 on LV function.18,19,40 As in previous studies, we observed an immediate improvement of FS and Vcf after acute administration of Ucn2, indicating potent inotropic effects. After 5 weeks of treatment with Ucn2, both FS and Vcf were preserved in Ucn2-treated DSS rats, whereas we observed a significant decrease of Vcf in vehicle-treated animals. Moreover, E/A ratio was significantly increased in vehicle-treated DSS rats, indicating significantly impaired LV filling properties in these animals, whereas E/A ratio was preserved in Ucn2-treated DSS rats. Taken together, these data suggest that chronic administration of Ucn2 has beneficial acute and chronic effects on both systolic and diastolic function in hypertensive DSS rats and may prevent the deterioration of LV function observed in vehicle-treated animals.

Perspectives

In summary, our study demonstrates that administration of Ucn2 produces an immediate BP reduction in hypertensive rats. Chronic administration of Ucn2 BID for 5 weeks resulted in a significant and sustained BP reduction (without downregulation of CRFR2) compared with vehicle-treated animals and retarded the development of hypertension-induced LVH. Most importantly, chronic administration of Ucn2 in hypertensive DSS rats prevented the deterioration of LV function observed in vehicle-treated animals and seemed to beneficially affect LV geometry. Taken together, the findings of our study indicate that chronic CRFR2 stimulation is feasible, effective, and may represent a novel and attractive approach for antihypertensive therapy.

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Disclosures

J.R. and W.W.V. report patent and licensing income in the CRF field in accordance with Salk Institute policy. W.W.V. is a cofounder, consultant, equity holder, and member of the Board of Directors and Scientific Advisory Board of Neurocrine Biosciences Inc. The remaining authors report no conflicts.
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IMMEDIATE AND SUSTAINED BLOOD PRESSURE LOWERING BY UROCORTIN 2: A NOVEL APPROACH TO ANTIHYPERTENSIVE THERAPY?

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<table>
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<th>Oligonucleotide</th>
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| **β-Tubulin**  | For 5′-CCGGACAGTGTGGCAACCAGATCGG  
Rev 5′-TGGCCAAAAGGACCTGAGCGAACGG | 193 bp |
| **CRFR2**      | For 5′-TGAGAAACATCACGTGGTTCCT  
Rev 5′-GCGGCACCAGACCTCATT | 74 bp |
| **Ucn2**       | For 5′-CCTGGATGTCCCCATTGG  
Rev 5′-GATTCCTGGCAGCCTTGTTC | 68 bp |
| **BNP**        | For 5′-CCAGAACAATCCACGATGCA  
Rev 5′-GCCATTTTCTCTGACTTTTCTCTTA | 62 bp |

Sequences of oligonucleotides used for real-time PCR.
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<th>Vehicle</th>
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<td>DSR / Ucn2</td>
<td>22.33 ± 0.52</td>
<td>22.67 ± 0.82</td>
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Raw Ct values of β-tubulin mRNA levels (mean ± SD). DSS: Dahl salt-sensitive rats; DSR: Dahl salt-resistant rats; CRFR2: CRF receptor type 2; Ucn2: Urocortin 2.
Time course of heart rate measured immediately after initial application of urocortin 2 or vehicle (A) and at the trough point after one, two, and five weeks of b.i.d. treatment (B) in Dahl salt-sensitive rats (urocortin 2 (■), vehicle (□)) respectively Dahl salt-resistant rats (urocortin 2 (●), vehicle (○)).
Quantification of mRNA levels of CRF receptor 2 and urocortin 2 in left ventricular (A, B) or aortic tissue (C, D) from Dahl salt-sensitive (DSS, n=7) and Dahl salt-resistant (DSR, n=6) rats after five weeks of b.i.d. treatment with urocortin 2 (■) or vehicle (□).