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.

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.1 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.2 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.3–7 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.8 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-hypothalamic-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) 1,9 Ucn2 (human ortholog: stresscopin-related peptide10,11), and Ucn3 (human ortholog: stresscopin11,12), signaling through 2 G protein–coupled receptors, the CRF receptors (CRFR) 1 and 2, have been discovered. Although CRFR1 and CRFR2

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From the Department of Biomedicine (T.D., S.M.-B., K.B., C.M., D.J., M.B.), University of Basel and University Hospital Basel, Basel, Switzerland; Division of Cardiology (T.D., P.T.B.), University Hospital Basel, Basel, Switzerland; Clayton Foundation Laboratories (J.R., W.W.V.), Salk Institute, La Jolla, Calif; and the Institute of Molecular Medicine (K.L.P.), University of California San Diego, La Jolla. T.D. and S.M.-B. contributed equally to this study. Correspondence to Thomas Dieterle, Division of Cardiology, University Hospital Basel, Petersgraben 4, CH-4031 Basel, Switzerland. E-mail dieterlet@uhbs.ch © 2009 American Heart Association, Inc. Hypertension is available at http://hyper.ahajournals.org DOI: 10.1161/HYPERTENSIONAHA.108.125211
are found in the central nervous system, CRFR2 is particu-
larly abundant in the periphery, including the heart and
systemic vasculature.13-17 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 cardiomyopath-
ic 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
baseline (before the first injection of Ucn2 or vehicle [BL]) and at 5,
time points after application of pentobarbital. BP was measured at
vehicle, BP measurements, and echocardiographic tracings at equal
was carefully controlled and strictly adapted to the BW of the
rats/H11005 (n = 10 in each group). The amount of pentobarbital used for sedation
(pentobarbital 20 mg/kg of BW IP), spontaneously breathing animals
and function were performed simultaneously on slightly sedated
BP measurements and echocardiographic analysis of LV dimensions
Echocardiography and BP Measurements
Ucn2 was provided by Dr Jean Rivier (Salk Institute).

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
equally and served as normotensive controls. After 15
expressions (A/β) and expres-
ions of Ucn2 and CRFR2 at week 5 of treatment with Ucn2 or

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
(n = 10 in each group). The amount of pentobarbital used for sedation
was carefully controlled and strictly adapted to the BW of the
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. Echocardi-
ography 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 per-
formed 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 brady-
cardia. 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. Transmural 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 (PWth), 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 shorten-
ing (Vcf) as Vcf (circumferences/s)=(πLVEDD-πLVESD)/(ET×πLVESD).
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, respec-
tively. Real-time PCR was performed in an ABI 7500 Fast Sequence
Detection System (Applied Biosystems) using iTag 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 (Dy-
namic Microsystems Inc, version 8.0). All of the values are ex-
pressed as means±SDs. Comparisons of HW/BW ratios and expres-
sion 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 μg/kg IP) or during chronic treatment (2.5 μg/kg IP BID) are shown in Figure 1. In hypertensive DSS rats, Ucn2 caused an immediate reduction of systolic BP of 29 mm Hg at 5 minutes after the first injection (*P<0.05 versus vehicle), persisting for ≥30 minutes. No effects on BP were observed in vehicle-treated hypertensive DSS rats (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 μg/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 (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,28 as well as an overactivity of the sympathetic nervous system.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.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-
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.

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 artery and the coronary circulation of pigs. The endothelium-dependent component appears to act primarily via the release of endothelial NO, which, in turn, stimulates Ca\(^{2+}\)-activated K\(^+\) channels in vascular smooth muscle via cGMP-dependent mechanisms. Moreover, vasodilatory effects via p38 mitogen-activated protein kinase and the protein kinase A pathway may contribute to the vasodilatory effect of Ucn2. In addition, potent diuretic effects of Ucn2 have been described at least in an animal model of CHF 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, the study was conducted using the human form of Ucn2. Although the rat and human forms of Ucn2 share an 83% homology, 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, and exposure of cultured cardiac myocytes to Ucn2 and Ucn3 resulted in increased myocyte size and protein synthesis. 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 LVPWith, 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. 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 CRF2 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?

Thomas Dieterle¹,² *, Silvia Meili-Butz¹ *, Katrin Bühler¹, Christian Morandi¹, Dietlinde John¹, Peter T. Buser², Jean Rivier³, Wylie W. Vale³, Kirk L. Peterson⁴, Marijke Brink¹

¹ Department of Biomedicine, University of Basel and University Hospital Basel, Switzerland, ² Division of Cardiology, University Hospital Basel, Switzerland, ³ Clayton Foundation Laboratories, The Salk Institute, La Jolla, CA, USA, ⁴ Institute of Molecular Medicine, University of California San Diego, La Jolla, CA, USA

* TD and SMB contributed equally to this study.

Correspondence: Thomas Dieterle, M.D.
Division of Cardiology
University Hospital Basel
Petersgraben 4
CH-4031 Basel
Phone: +41-61-265-2525
Fax: +41-61-265-4598
E-mail: dieterlet@uhbs.ch
### Table S1

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Amplicon length</th>
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</table>
| **β-Tubulin**   | For 5'-CCGGACAGTGTGGCAACCAGATCGG  
                 | Rev 5'-TGGCCAAAAGGACCTGAGCGAACGG | 193 bp |
| **CRFR2**       | For 5'-TGAGAAACATCACGTGGTTCTCTT  
                 | Rev 5'-GCGGCACCAGACCTCATT | 74 bp |
| **Ucn2**        | For 5'-CCTGGATGTCCCCATTGGG  
                 | Rev 5'-GATTCCTGGCAGCGTTTC | 68 bp |
| **BNP**         | For 5'-CCAGAACAATCCACGATGCA  
                 | Rev 5'-GCCATTTTCCTCTGACTTTTCTCTTA | 62 bp |

Sequences of oligonucleotides used for real-time PCR.
Table S2

<table>
<thead>
<tr>
<th>Rat strain / analysed gene</th>
<th>Ucn2</th>
<th>Vehicle</th>
</tr>
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<tbody>
<tr>
<td>DSS / CRFR2</td>
<td>26.13 ± 0.16</td>
<td>26.34 ± 0.84</td>
</tr>
<tr>
<td>DSR / CRFR2</td>
<td>26.79 ± 0.53</td>
<td>27.17 ± 0.80</td>
</tr>
<tr>
<td>DSS / Ucn2</td>
<td>21.63 ± 0.18</td>
<td>21.90 ± 0.77</td>
</tr>
<tr>
<td>DSR / Ucn2</td>
<td>22.33 ± 0.52</td>
<td>22.67 ± 0.82</td>
</tr>
</tbody>
</table>

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 (○)).
Figure S2

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 (□).