Elevated plasma levels of human urotensin-II immunoreactivity in congestive heart failure

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HUMAN UROTENSIN-II (hU-II) is the most potent endogenous cardio stimulant identified to date. We therefore determined whether hU-II has a possible pathological role by investigating its levels in patients with congestive heart failure (CHF).

Blood samples were obtained from the aortic root, femoral artery, femoral vein, and pulmonary artery from CHF patients undergoing cardiac catheterization and the aortic root from patients undergoing investigative angiography for chest pain who were not in heart failure. Immunoreactive hU-II (hU-II-ir) levels were determined with radioimmunoassay.

hU-II-ir was elevated in the aortic root of CHF patients (230.9 ± 68.7 pg/ml, n = 21; P < 0.001) vs. patients with nonfailing hearts (22.7 ± 6.1 pg/ml, n = 18). This increase was attributed to cardiopulmonary production of hU-II-ir because levels were lower in the pulmonary artery (38.2 ± 6.1 pg/ml, n = 21; P < 0.001) than in the aortic root. hU-II-ir was elevated in the aortic root of CHF patients with nonischemic cardiomyopathy (142.1 ± 51.5 pg/ml, n = 10; P < 0.05) vs. patients with nonfailing hearts and coronary artery disease (27.3 ± 12.4 pg/ml, n = 7) and CHF patients with ischemic cardiomyopathy (311.6 ± 120.4 pg/ml, n = 11; P < 0.001) vs. patients with nonfailing hearts and coronary artery disease (19.8 ± 6.6 pg/ml, n = 11). hU-II-ir was significantly higher in the aortic root than in the pulmonary artery and femoral vein, with a nonsignificant trend for higher levels in the aortic root than in the femoral artery. The findings indicated that hU-II-ir is elevated in the aortic root of CHF patients and that hU-II-ir is cleared at least in part from the microcirculation.

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cardiomyopathy associated with nonischemic etiologies including idiopathic dilated cardiomyopathy (n = 7) and alcoholic (n = 1), viral (n = 1), and hypertrophic obstructive (n = 1) cardiomyopathy. Drug treatment for the two cohorts is described in Table 1. Sites of clearance of hU-II-ir were 1) cardiomyopathy. Drug treatment for the two cohorts is

Table 1. Drug treatment for patients with nonfailing hearts and congestive heart failure

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>Nonfailing</th>
<th>CHF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>β-Adrenoceptor antagonist</td>
<td>14</td>
<td>77.8</td>
</tr>
<tr>
<td>L-type Ca²⁺ antagonist</td>
<td>1</td>
<td>5.6</td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>9</td>
<td>50.0</td>
</tr>
<tr>
<td>ANG II receptor antagonist</td>
<td>1</td>
<td>5.6</td>
</tr>
<tr>
<td>Diuretic</td>
<td>1</td>
<td>5.6</td>
</tr>
<tr>
<td>Hypolipidemic</td>
<td>9</td>
<td>50.0</td>
</tr>
<tr>
<td>Antiangina</td>
<td>9</td>
<td>50.0</td>
</tr>
<tr>
<td>Cardiac glycoside</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Antiarrhythmic</td>
<td>1</td>
<td>5.6</td>
</tr>
</tbody>
</table>

n, Number of patients treated. Total number of patients: nonfailing hearts, 18; congestive heart failure (CHF), 21. ACE, angiotensin-converting enzyme. * P = 0.0002, † P < 0.0001, ‡ P = 0.0006 compared with the nonfailing cohort (Fisher’s exact test).

Samples were spun (1,600 g, 15 min, 4°C), plasma was extracted, and hU-II-ir levels were measured by radioimmunoassay according to the manufacturer’s instructions (Phoenix Pharmaceuticals). In addition, cross-reactivity of the assay to a pro-hU-II fragment, endothelin-1 (ET-1), angiotensin II, and norepinephrine was tested. Briefly, plasma was acidified with 1% trifluoroacetic acid, spun (14,000 g, 20 min, 4°C), and loaded on to a Sep-Pak C18. Samples were eluted with 60% acetonitrile in 1% trifluoroacetic acid and dried with a Speed Vac Concentrator (Savant). Dried plasma extracts, synthetic hU-II standards, a 25-amino acid carboxy-terminal sequence of the hU-II prohormone (containing the mature sequence (residues in italics): SHLLARIWKPYKRETPDCFWKYCV), ET-1, angiotensin II, and l-norepinephrine were diluted with RIA buffer and incubated with primary antibody (17 h, 4°C), 125I-labeled hU-II (10,000 cpm/0.1 ml; 24 h, 4°C), and then goat anti-rabbit IgG and normal rabbit serum (1.5 h, 23°C). The experiment was terminated by addition of RIA buffer, samples were spun (1,700 g, 20 min, 4°C), and pellets were counted (LKB Wallac 1272 Clinigamma). Data were corrected for efficiency of peptide extraction (40.2%).

Liquid chromatography-mass spectrometry. Gradient high-performance liquid chromatography was used to analyze synthetic mature hU-II and plasma samples. Plasma was extracted (see Radioimmunoassay), reconstituted in 0.1% trifluoroacetic acid, and chromatographed on an Agilent Zorbax 5-μm SBC-18 column (2.1 × 50 mm) at a flow rate of 0.3 ml/min with solvent A (0.1% formic acid, 2 min), 100% solvent A ramping to 90% solvent B (0.1% formic acid, 90% acetonitrile in distilled water) until 10 min, held at 90% solvent B for 2 min, returned to 100% solvent A in 6 s, and washed with 100% solvent A for a further 6 min. Solvents for the binary gradient were delivered with Shimadzu LC10 AT VP liquid chromatography (LC) pumps that were controlled with a SCL10A VP Shimadzu system controller. Solvents were degassed with a DGU12A Shimadzu degasser. Samples were injected onto the mass spectrometer (MS) with an Agilent 1100 series auto injector. MS analyses were carried out with a PE Sciex API3000 LC-MS-MS system. Samples were ionized by positive-ion electrospray atmospheric pressure ionization. The ion spray, orifice (Or), and ring voltages were 5,200, 35, and 225 V, respectively. The quadrupole zero (Q0) value was −10 V, and the declustering potential was 45 V (Or − Q0).

Human atrial contraction studies. Tissue bath studies were carried out as previously described (10). Briefly, human

Table 2. Hemodynamic data for patients with nonfailing hearts and congestive heart failure patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nonfailing</th>
<th>CHF</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>All patients</td>
<td>CAD</td>
</tr>
<tr>
<td>n (M/F)</td>
<td>18(15/3)</td>
<td>11(11/0)</td>
</tr>
<tr>
<td>Age, yr</td>
<td>59.4 ± 2.9</td>
<td>62.7 ± 3.6</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28.7 ± 1.4</td>
<td>30.2 ± 1.9</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>67.3 ± 2.3</td>
<td>63.4 ± 2.9</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>63.4 ± 2.9</td>
<td>62.0 ± 3.4</td>
</tr>
<tr>
<td>PCWP, mmHg</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CI, l/min·m⁻²</td>
<td>2.3 ± 0.6</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>SC, mol/C₄</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE for n patients. CAD, coronary artery disease; ICM, ischemic cardiomyopathy; M/F, male/female; BMI, body mass index; LVEF, left ventricular ejection fraction; HR, heart rate; PCWP, pulmonary capillary wedge pressure; CI, cardiac index; SC, serum creatinine. PCWP and CI were not determined (ND) in nonfailing individuals; however, previously reported control values are PCWP 2–12 mmHg (7) and CI, 2.8–4.2 l/min·m⁻² (7). * P < 0.001, † P < 0.05, ‡ P < 0.001 compared with all patients in the nonfailing cohort (unpaired t-test).
right atrial trabeculae were obtained from patients undergoing coronary artery bypass surgery and were set up in 50-mL baths containing modified Krebs solution at 37°C. Cumulative concentration response curves were carried out for mature synthetic hU-II (20 pM–20 nM) and pro-hU-II fragment (20 pM–20 nM) in the presence of 200 nM l-propranolol to block endogenous l-norepinephrine stimulation of \( \beta_1 \)- and \( \beta_2 \)-adrenoceptors. Mature synthetic hU-II (20 nM) was added to tissues that were exposed to the prohormone fragment. Maximal receptor-independent responses were determined by increasing the Ca\(^{2+}\) concentration to 9.25 mM, and curves were corrected for time-dependent fade with nonstimulated control tissues.

**Statistics.** Data were normalized by log transformation and analyzed with one-way ANOVA or Student’s \( t \)-test. Data sets showing a significant difference by one-way ANOVA (\( P < 0.05 \)) were examined with the Tukey multiple-comparisons test. Male-to-female ratios and drug therapies were compared with a Fisher’s exact test. Pearson’s correlation coefficient was used to examine the association between hemodynamic variables and plasma hU-II-ir levels. Values are expressed as means ± SE.

**Reagents.** The hU-II radioimmunoassay kit and C\(_{18}\) Sep-Pak columns were from Phoenix Pharmaceuticals. ET-1, hU-II, and the pro-hU-II fragment were synthesized by Auspep (Parkville, Australia).

**RESULTS**

**Elevated plasma hU-II-ir levels in human heart failure.** Aortic root plasma hU-II-ir levels were markedly elevated in CHF patients compared with patients with nonfailing hearts (Fig. 1A; \( P < 0.001 \), 1-way ANOVA). This increase was attributed to cardiac and/or pulmonary production of the peptide, because concentrations were higher in the aortic root than the pulmonary artery in CHF patients (Fig. 1A; \( P < 0.001 \), 1-way ANOVA). LVEF determined in all patients was inversely correlated with hU-II-ir levels (Fig. 2, \( r = -0.61; P < 0.0001 \), Pearson’s correlation coefficient) and was lower in the CHF cohort compared with patients with nonfailing hearts (Table 2; \( P < 0.0001 \), unpaired \( t \)-test). A correlation was not observed between hU-II-ir levels and pulmonary wedge pressure or cardiac index, although this was determined in the CHF cohort only because values for pulmonary wedge pressure and stroke volume were not obtained for patients with nonfailing hearts. Serum creatinine levels (\( P = 0.0003 \)) and heart rate (\( P = 0.029 \)) were higher in patients with CHF than in patients with nonfailing hearts (unpaired \( t \)-test; Table 2). A correlation was not observed between hU-II-ir levels and creatinine levels or heart rate (\( P > 0.05 \), Pearson’s correlation coefficient). Plasma hU-II-ir levels were not correlated with NYHA class (\( P > 0.05 \), 1-way ANOVA).

Data obtained from CHF patients were separated according to ischemic or nonischemic etiology. Plasma hU-II-ir levels were higher in the aortic root of patients with ischemic cardiomyopathy compared with patients with nonfailing hearts with coronary artery disease (\( P < 0.001 \), 1-way ANOVA) and in patients with nonischemic cardiomyopathy compared with patients with nonfailing hearts and normal coronary arteries (\( P < 0.05 \), 1-way ANOVA; Fig. 1B). A cardiopulmonary gradient for hU-II-ir was observed in ischemic cardiomyopathy patients (\( P < 0.01 \); 1-way ANOVA), with a nonsignificant trend observed in patients with nonischemic cardiomyopathy. There was no difference between hU-II-ir levels in the aortic root of patients with ischemic and nonischemic cardiomyopathies or between patients with nonfailing hearts with or without coronary artery disease.

To investigate sites of clearance or degradation of hU-II-ir from the peripheral circulation, additional

**Fig. 1.** Immunoreactive human urotensin-II (hU-II-ir) levels in patients with nonfailing hearts (\( n = 18 \)) and patients with congestive heart failure (CHF; \( n = 21 \)). A: plasma hU-II-ir levels in the aortic root (filled bars) and pulmonary artery (open bar) of patients with CHF and nonfailing hearts. B: plasma hU-II-ir levels in the aortic root (filled bars) and pulmonary artery (open bars) of patients with ischemic (\( n = 11 \)) and nonischemic (\( n = 10 \)) cardiomyopathy compared with patients with nonfailing hearts with (+CAD, \( n = 11 \)) or without (–CAD, \( n = 7 \)) coronary artery disease. C: plasma hU-II-ir levels in the aortic root, femoral artery, femoral vein, and pulmonary artery from patients with CHF (\( n = 10 \)). Values are means ± SE. \(* P < 0.05\), 1-way ANOVA on log-transformed data.
blood samples were taken from the femoral artery and femoral vein of patients with CHF. hU-II-ir levels were higher in the aortic root than in the femoral vein (P < 0.05) and pulmonary artery (P < 0.01) but were not different between the aortic root and femoral artery (P > 0.05, 1-way ANOVA; Fig. 1C).

Immunoreactive hU-II peptides. Experiments were carried out to verify the recognition of mature synthetic hU-II with radioimmunoassay. The antibody, which was raised against the mature hU-II sequence, recognized synthetic mature hU-II with a high sensitivity of detection (1–10 pg/ml). Although the antibody did not cross-react with ET-1 (100 nM), angiotensin II (124 pM), l-norepinephrine (14 nM) (this study; not shown) or C-type natriuretic peptide-22, CGRP, adrenomedullin, bradykinin, brain natriuretic peptide (BNP)-45, BNP-32, proadrenomedullin N-terminal 20-peptide, or α-atrial natriuretic peptide-28 (Phoenix Pharmaceuticals data sheet), it exhibited 29.0 ± 11.7% (n = 4) cross-reactivity with the pro-hU-II fragment (see MATERIALS AND METHODS). This raised the possibility that the circulating peptide may be the prohormone rather than the mature peptide. Consistent with this hypothesis, no compound with the same characteristics as mature hU-II was detected in plasma samples by LC-MS. The sensitivity of detection of hU-II with this method was 64 pg.

Prohormone-converting enzyme activity is required for biological activity. Contractile studies were carried out to compare the cardiac effects of the pro-hU-II fragment and mature hU-II. Although hU-II (20 pM–20 nM) caused a concentration-dependent increase in force of contraction of human right atrial trabeculae from nonfailing hearts (pEC50 = 9.5 ± 0.02, maxima = 30.4 ± 1.6% of response to 9.25 mM Ca2+; n = 4 tissues/3 patients), tissues from the same patients were largely unresponsive to the pro-hU-II fragment (20 pM–20 nM; n = 5 tissues/3 patients) (Fig. 3). Tissues exposed to the pro-hU-II fragment were responsive to 20 nM mature hU-II when added at the completion of the concentration-response curve (maxima = 27.5 ± 3.6% of response to 9.25 mM Ca2+), demonstrating preserved muscle viability. These findings indicate that if pro-hU-II or a carboxy-terminal fragment that is ≥25 amino acids in length is the circulating hormone in CHF patients, an active prohormone-converting enzyme would be required to produce a biologically active peptide.

DISCUSSION

This study revealed markedly elevated plasma hU-II-ir levels in CHF patients with ischemic and nonischemic etiologies compared with patients with nonfailing hearts. This increase did not appear to be related to the presence of coronary artery disease because plasma hU-II-ir levels in patients with nonfailing hearts but with coronary artery disease were not elevated.

Plasma hU-II-ir levels in CHF patients were higher in the aortic root than in the pulmonary artery, consistent with cardiopulmonary production. The cardiopulmonary gradient for hU-II-ir observed in this study is in line with increased myocardial expression of hU-II peptide in left ventricle compared with right ventricle obtained from patients with CHF secondary to ischemic heart disease (3), and this may therefore represent a site of production for the hU-II-ir that was detected in the plasma. Although mRNA encoding the U-II precursor was absent in lung (1, 2), we cannot exclude the possibility that hU-II-ir is secreted from the lungs under pathophysiological conditions such as CHF. The increase in hU-II-ir in the aortic root is in line with other studies that showed increased hU-II-ir in the peripheral circulation of patients with CHF (8, 9).

Interestingly, unlike the correlation observed for circulating ET-1 levels and NYHA function class (13), no correlation was observed for hU-II-ir and NYHA functional class in the CHF cohort (this study; Ref. 8).

Patients with renal dysfunction had elevated plasma hU-II levels, and this was attributed to reduced capacity for peptide excretion or increased hU-II production (11). However, the elevated hU-II-ir levels in CHF patients, of whom only 7 showed evidence of renal dysfunction (creatinine = 0.16 ± 0.01 mmol/l), cannot be explained by renal disease because elevated hU-II-ir levels were detected in the remaining 14 CHF patients.
Plasma levels of urotensin-II were measured in the aortic root samples. Plasma urotensin-II levels in patients treated with ACE inhibitors and with nonfailing hearts (24.7 ± 7.7 pg/ml, n = 9) were different from those in patients treated with ACE inhibitors and with CHF (230.9 ± 68.7 pg/ml, n = 21; P < 0.0001). None of the patients with nonfailing hearts was treated with cardiac glycosides; however, no difference in urotensin-II levels were observed for CHF patients treated with (190.0 ± 63.6 pg/ml, n = 10) or without (263.0 ± 120.0 pg/ml, n = 11; P = 0.89) cardiac glycosides. Only 1 patient with a nonfailing heart was treated with a diuretic compared with 20 patients with CHF. The plasma level of urotensin-II in this patient (29 pg/ml) was within the range of levels determined in patients with nonfailing hearts (3.2–53.9 pg/ml). Therefore, although the management of patients with CHF was different from that of patients with nonfailing hearts, these differences were not associated with urotensin-II levels in the aortic root.

It is not known whether the peptide contributes to the pathology of cardiac disease or whether it is a marker of the disease process. Although urotensin-II is predicted to produce transient benefits associated with improved cardiac output in CHF patients, long-term cardiostimulation would be detrimental if it increased oxygen consumption in the face of reduced myocardial energy efficiency that is associated with heart failure (15). U-II stimulated cardiomyocyte hypertrophy (17) and α1(I) and α1(III) procollagen gene expression in fibroblasts (14) in rat neonatal cell cultures, and additional harmful effects may therefore be associated with a role of urotensin-II in myocardial hypertrophy and remodeling. Plasma levels of l-norepinephrine and ET-1 are also elevated in heart failure, indicating that CHF triggers events leading to secretion of multiple cardiactive agents into the circulation that in combination may contribute to the progression of cardiac disease.

Plasma levels of urotensin-II were measured in the aortic root, femoral artery, femoral vein, and pulmonary artery to examine possible sites of peptide clearance. U-II levels were lower in the pulmonary artery and femoral vein than in the aortic root, with a nonsignificant trend toward a reduction in the femoral artery. The trend for decreased urotensin-II levels in the femoral artery compared with the aortic root could indicate that the aorta contributes in part to the efficient clearance of urotensin-II. The regional differences in urotensin-II levels were similar to gradients reported for ET-1, where lower plasma ET-1 levels were detected in the femoral vein than in the aortic root and femoral artery (12, 13) and suggest a possible role of the microcirculation in urotensin-II clearance.

Interestingly, the antibody used to detect mature urotensin-II also cross-reacted with a pro-urotensin-II fragment, raising the possibility that the prohormone could be the endogenous circulating peptide. Consistent with this, no compound with the same characteristics as synthetic mature urotensin-II was detected by mass spectrometry. Indeed, this may explain differences observed between our findings and a study by Dschietzig et al. (4), which showed no increase in urotensin-II levels in CHF patients. The high molecular weight of pro-urotensin-II (124- and 139-amino acid residue splice variants have been identified) confers low sensitivity of detection by MS, and although we speculate that pro-urotensin-II may be the circulating hormone, further investigation is required to prove this hypothesis. If the prohormone is secreted into the circulation in CHF patients, then the presence of an active urotensin-converting enzyme may be crucial for biological activity. Consistent with this hypothesis, tissue bath studies revealed that human atrium was largely unresponsive to the pro-urotensin-II fragment (20 pM–20 nM) whereas mature urotensin-II potently increased force of contraction.

In conclusion, this study provides evidence for elevated plasma urotensin-II levels in patients with ischemic and nonischemic cardiomyopathy compared with patients with nonfailing hearts with or without coronary artery disease. The increased levels of urotensin-II are rapidly cleared from the circulation, suggesting local autocrine or paracrine effects of the peptide.

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DISCLOSURES

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