Direct Actions of Urotensin II on the Heart
Implications for Cardiac Fibrosis and Hypertrophy

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Abstract—Urotensin II (UII) is a somatostatin-like peptide recently identified as a potent vasoconstrictor. In this study, we examined whether UII promotes cardiac remodeling through nonhemodynamic effects on the myocardium. In a rat model of heart failure after myocardial infarction (MI), increased UII peptide and UII receptor protein expression was observed in both infarct and noninfarct regions of the left ventricle compared with sham. Moreover, post-MI remodeling was associated with a significant 75% increase in UII receptor gene expression in the heart (P<0.05 versus sham controls), with this increase noted in both regions of the left ventricle. In vitro, UII (10⁻⁷ mol/L) stimulation of neonatal cardiac fibroblasts increased the level of mRNA transcripts for procollagens α(I), α(III), and fibronectin by 139±15% (P<0.01), 59±5% (P<0.05), and 141±14% (P<0.01), respectively, with a concomitant 23±2% increase in collagen peptide synthesis as determined by ³H-proline incorporation (P<0.01). UII had no effect on cellular hypertrophy, as determined by changes in total protein content in isolated neonatal cardiomyocytes. However, expression of recombinant rat UII receptor in neonatal cardiomyocytes resulted in significant UII-dependent activation of hypertrophic signaling as demonstrated by increased total protein content (unstimulated, 122.4±4.0 μg/well; rat UII, 147.6±7.0 μg/well; P<0.01) and activation of the hypertrophic phenotype through Gq- and Ras-dependent pathways. These results indicate that, in addition to potent hemodynamic effects, UII may be implicated in myocardial fibrogenesis through increased collagen synthesis by cardiac fibroblasts and may also be an important determinant of pathological cardiac hypertrophy in conditions characterized by UII receptor upregulation. (Circ Res. 2003;93:246-253.)

Key Words: urotensin | myocardial infarction | remodeling | collagen | hypertrophy

Left ventricular dysfunction, regardless of the underlying etiology, is associated with activation of various neuro-hormonal systems that compensate for the consequent loss of ventricular function and peripheral homeostasis. These include the renin-angiotensin and endothelin systems, which act to restore the early decline in cardiac output through positive inotropic effects and peripheral vasconstriction. Although these effects may be beneficial in the short term, prolonged activation, both local and systemic, may additionally contribute to worsening of contractile function through adverse cardiac remodeling, a process characterized by necrosis,1 myocyte hypertrophy,2 inflammation,3 and the pathological accumulation of extracellular matrix material within the interstitium (fibrosis).4

More recently, the vasoconstrictor peptide urotensin II (UII) has emerged as a likely contributor to cardiovascular physiology and pathology. UII is a somatostatin-like cyclic peptide synthesized by proteolytic cleavage from a precursor molecule, prepro-UII, and has recently been identified as a potent vasoconstrictor.5 UII has been identified within the heart,6 which is also an abundant site of UII receptor (UIIR) expression.5 Acute UII infusion into nonhuman primates results in severe myocardial depression, characterized by bradycardia and reduced stroke volume and contractility,5 suggesting a functional role for this peptide within the heart. However, the role of UII within the myocardium remains poorly understood, particularly in the setting of disease.

Thus, UII may be an important determinant of cardiac dysfunction via additional nonhemodynamic effects on the myocardium, analogous to those previously described for other vasoconstrictor peptides such as angiotensin II (Ang II) and endothelin 1 (ET-1). Accordingly, this study sought to determine whether UII has direct effects on isolated cardiac cell types in vitro, as well as examining the expression of UII in an experimental model of left ventricular remodeling after myocardial infarction (MI) in the rat. The data demonstrate that UII peptide and receptor expression is upregulated within injured myocardium. Moreover, we show for the first time that UII is profibrogenic in cardiac fibroblast cultures and can regulate hypertrophic signaling in cardiomyocytes.
Materials and Methods

Left Ventricular MI in the Rat

The animal model, as previously described,\(^7\) conforms to the Guide for the Care and Use of Laboratory Animals published by the NIH (NIH publication No. 85-23, revised 1996). At 1 or 2 weeks after surgery (sham, n=5; left ventricular MI [LVMI], n=5), hemodynamic measurements were performed\(^7\) and then whole hearts were excised, embedded into OCT embedding compound, and snap frozen over liquid nitrogen for subsequent immunohistochemical analysis and total RNA isolation. Infarct size was determined on cryostat sections as previously described\(^7\) in detail.

Immunohistochemistry

Immunoreactive UII was assessed using an avidin-biotin technique on either snap-frozen tissue sections postfixed with 4% paraformaldehyde or 10% buffered formalin-fixed, paraffin-embedded tissue sections.\(^8\) Sections of the left ventricle (LV) were incubated overnight at 4°C with rabbit anti-human UII antibody (a kind gift of Dr S. Douglas, Glaxo SmithKline, King of Prussia, Pa), rat anti-GPR14 antibody (Alpha Diagnostics, San Antonio, Tex), and mouse anti-human mast cell chymase (Chemicon International, Temecula, Calif). Cross reactivity of the UII antibody was absent or minimal against a range of peptide ligands, as previously described.\(^9\) Negative controls represented either the absence of primary antibody or an irrelevant antibody control.

Neonatal Cardiomyocyte and Fibroblast Cultures

Neonatal cardiomyocytes and fibroblasts, isolated from the hearts of 1-day-old Sprague-Dawley rat pups, were seeded onto 12-well plates at 0.125\(^\times\)10\(^4\) cells/mm\(^2\) (high density) in MEM supplemented with 10% FBS and bromodeoxyuridine.\(^10\) After 16 hours, the cells were placed into serum-free defined medium\(^10\) and maintained in this medium thereafter. To prevent spontaneous contraction, cultures...
were maintained in medium containing 50 mmol/L KCl. Neonatal cardiac fibroblasts were purified by differential plating and used at passage 1 for all experiments.

Measurement of Collagen Synthesis by 
3H-Proline Incorporation

In vitro collagen synthesis by neonatal cardiac fibroblasts was determined on confluent cultures by 3H-proline incorporation as previously described.11

Northern Blot Analysis

Levels of procollagen α(I) and α(I) and fibronectin mRNA transcripts were determined by Northern blot analysis as previously described7 using 32P-labeled cDNA fragments complementary to procollagen α(I), α(I),7 and rat fibronectin generated by reverse transcription–polymerase chain reaction (RT-PCR) from infarcted rat cardiac tissue and spanning positions 1002 to 1578 of the published sequence.12

RNase Protection Analysis

Level of UIIR and AT1a receptor mRNA transcripts was measured by RNase protection analysis as previously described.13 cRNA probes were generated from a 449-bp fragment of the rat UIIR cDNA corresponding to nucleotides 799 to 1248 and a 618-bp fragment of the rat AT1a receptor cDNA corresponding to nucleotides –52 to +566. As a control, a 177-nucleotide GAPDH probe was cohybridized in each reaction.

Reverse Transcription–Polymerase Chain Reaction

Rat UIIR gene transcripts were amplified by RT-PCR from DNAse I-treated (0.2 U/μL), reverse-transcribed mRNA from neonatal cardiomyocyte and fibroblasts using sense (5'-GGGCATGGTGGG-AAAATGTA-3') and antisense (5'-CGCCGTGTCTGCTTGAAAG-3') primers corresponding to positions 427 and 1014, respectively, of the published sequence.5

Quantitative Real-Time PCR

UII gene expression was measured and quantified using the GeneAmp 5700 Sequence Detector (PE Biosystems) according to the manufacturer’s instructions. Primers were obtained from Sigma-Aldrich. The fluorogenic probe (Applied Biosystems) includes a fluorescence reporter (6-carboxyfluorescein [FAM]) at the 5' end and a fluorescent quencher (6-carboxytetramethylrhodamine [TAMRA]) at the 3' end. A commercial, predeveloped 18S control kit labeled with the fluorescent reporter dye [VIC] on the 5' end and the quencher [TAMRA] on the 3' end (PE Biosystems) was used as the endogenous control. The 25-μL PCR mixture contains 12.5 μL of Taqman Universal PCR Master Mix, 500 nmol/L primers (forward and reverse), 100 nmol/L of Taqman probe, and 1 μL of cDNA template. PCR was performed at 50°C for 2 minutes and 95°C for 10 minutes and then run for 50 cycles at 95°C for 15 seconds and 60°C for 1 minute. Results were expressed as the ratio of UII to 18S relative to sham hearts, which were arbitrarily assigned a value of 1.

Transient Transfection and Reporter Assays

Cardiomyocytes were transfected with 0.45 μg of myosin light chain (MLC)-2v-CAT, atrial natriuretic peptide (ANP) 328-Luc, or α skeletal actin (α-skACT)-CAT, together with 0.45 μg of the appropriate expression vector for the rat UIIR (rUIIR) (pKc/CMV-rUIIR) using Lipofectamine.15 All plates received equivalent amounts of DNA (0.9 μg) by cotransfection, where necessary, with the appropriate empty expression vector. Twenty-four hours after transfection, cells were stimulated with vehicle, UII, or phenylephrine (PE) (for appropriate expression vector) for 1 minute. Results were expressed as the ratio of UII to 18S relative to sham hearts, which were arbitrarily assigned a value of 1.

Generation of Recombinant Adenovirus

Recombinant adenovirus directing the expression of the rat UIIR (Ad-rUIIR) was generated by bacterial homologous recombination, as described.16,17 Twenty-four hours after plating, cells were infected with purified virus at a multiplicity of infection (MOI) of 12.5 to 300 in defined medium as indicated.17 An MOI of 100 corresponds to 1 × 10^6 PFU of virus, which infected greater than 95% of 1 × 10^6 myocytes and fibroblasts as defined by green fluorescent protein fluorescence. After 24 hours of infection, the medium was replaced and cells were assayed in accordance with the previously outlined methodologies.

Urotensin II Receptor Binding Assay

Expression of UII receptors in cultures of neonatal cardiomyocytes and fibroblasts after infection with adenovirus (Ad-rUIIR) was determined using a whole–cell binding assay18 with radiolabeled ([125I])-rat UII. [125I]-rat UII was prepared (specific activity ~1000 Ci/mmol, Austin Biomedical Services) from HPLC purified rat UII peptide (kindly synthesized, cyclized, purified, and authenticated using mass spectrometry by K. Stewart, Monash University and C. Hamilton, Baker Medical Research Institute).

Figure 2. Immunohistochemistry using anti-GR14 antibody (magnification ×380) demonstrated that UIIR was not detectable in the myocardium of sham rats (A), whereas after MI, UIIR was localized to myocytes (B), endothelial cells (C), and fibroblasts (C, arrow).
Statistical Analysis
Nonparametric analysis by Mann-Whitney U test was applied to all data sets, with a P<0.05 representing statistical significance.

Results
Myocardial Expression of UII and UIR After MI
MI rats had moderate to large infarcts (34.7±0.5% of length of LV epicardial and endocardial circumferences). In comparison with sham, MI animals demonstrated characteristic hemodynamic changes, with a markedly elevated LV end diastolic pressure (19.4±0.03 versus 9.2±0.02 mm Hg, P<0.05), reduced LV dP/dt max (5.4±0.8 versus 8.3±0.7 mm Hg/ms, P<0.05), and reduced mean arterial pressure (86±4 versus 111±5 mm Hg, P<0.05).

UII Receptor
RNase protection analysis demonstrated a significant 80±11% increase in UIR mRNA transcripts in whole heart.
by 1 week after MI compared with sham controls (Figure 1A). In comparison, AT1a receptor mRNA was increased by 175±35%. Additional analysis by RT-PCR showed that both neonatal cardiomyocyte and nonmyocyte populations (predominantly fibroblasts) constitutively express rat UIIR mRNA transcripts (Figure 1B).

Assessment of the distribution of this increase in UIIR expression was performed by RNase protection analysis. This demonstrated increased expression of UIIR in both the infarct and noninfarct zones of the LV as well as in right ventricle (Figure 1C), ANP expression was also measured as a marker gene known to be activated in the post-MI setting (Figure 1C).

Immunohistochemistry using anti-GR14 antibody demonstrated that UIIR was not detectable in the myocardium of sham rats (Figure 2A) whereas post-MI UIIR was localized to myocytes (Figure 2B), endothelial cells, and fibroblasts (Figure 2C).

**UII Peptide**

In normal and sham-operated rat heart, UII peptide was barely detected and immunolocalized predominantly to the vasculature (Figure 3a). In contrast, animals with MI demonstrated increased cellular expression of UII peptide within the left ventricular infarct region (Figures 3c and 3d) and increased diffuse staining within the interstitium of peri-infarct regions (Figures 3e and 3f). Additional analysis revealed that cellular UII expression within the infarct region was localized to chymase-positive mast cells (Figures 3g and 3h). Pre-pro UII peptide gene expression as determined by quantitative real-time PCR revealed increased UII mRNA in both infarct and noninfarct zones of the LV compared with sham animals (Ln-transformed fold increases compared with sham: right ventricle, 4.29; left ventricular noninfarct zone, 5.98; left ventricular infarct zone, 4.15). No significant correlations were observed between UII or UIIR expression and hemodynamic parameters or infarct size.

**Effect of UII on Collagen Synthesis by Neonatal Cardiac Fibroblasts In Vitro**

Compared with medium alone, UII stimulated an increase in fibronectin, α1(I), and α1(III) procollagen mRNA transcripts in neonatal cardiac fibroblasts (Figure 4A). This was associated with a concomitant increase in collagen synthesis, whereas there was no discernible synergistic effect with Ang II (Figure 4B).

Introduction of recombinant rUIIR introduced into cells using adenoviral vectors resulted in a dose-dependent increase in [125I]-UII binding compared with noninfected cultures (Figure 5A). Moreover, cells infected with even the lowest dose of Ad-rUIIR (MOI 12.5) demonstrated an even greater profibrogenic response to exogenous UII (Figure 5B) than noninfected cells. These results clearly demonstrate that modest overexpression of the UIIR enhances the profibrogenic stimulus of UII in neonatal cardiac fibroblasts. UII lacked a mitogenic effect in primary neonatal cardiac fibroblasts or in cells overexpressing recombinant rUIIR (data not shown).

![Figure 4](http://circres.ahajournals.org/)

**Figure 4.** UII stimulates matrix synthesis by neonatal rat cardiac fibroblasts in vitro. A, Fibronectin and procollagen αI(I) and αI(III) mRNA were measured by Northern blot analysis after 24-hour stimulation with ET-1 (10⁻⁷ mol/L) and UII (10⁻⁷ mol/L). Data represent samples analyzed over 4 separate experiments, and the results are expressed as a mean percentage of unstimulated controls (C) (±SEM) relative to GAPDH (n=12). B, Collagen peptide synthesis was determined by standard [³H-L-proline incorporation after 48-hour stimulation with Ang II (10⁻⁷ mol/L), UII (10⁻⁷ mol/L), or combined stimulation in DMEM-F12 medium supplemented with 0.15 mmol/L ascorbic acid. Data represent samples analyzed over 3 separate experiments, and the results are expressed as a mean percentage of unstimulated controls (C) (±SEM) (n=9). *P<0.05, **P<0.01 vs unstimulated controls.

**Effect of UII on Cardiac Myocyte Hypertrophy In Vitro**

In contrast to the hypertrophic stimulus of ET-1 and PE (total protein content [μg/well]; unstimulated, 10.2±3.0; ET-1, 16.0±1.9; PE, 13.7±0.4; P<0.01), UII had no effect on myocyte hypertrophy in high-density cultures (rUII [10⁻⁷ mol/L], 10.5±0.8 μg). In each case, the inability of UII to stimulate hypertrophy was not attributable to loss of cell number, because DNA content was unchanged (unstimulated, 2.98±0.1; ET-1, 3.11±0.3; PE, 3.05±0.2; rUII, 3.12±0.2). In support of the hypothesis that levels of rUIIR in primary neonatal cardiomyocytes may be below the threshold required for a hypertrophic response, we found that in cells transiently transfected with recombinant rUIIR, UII stimulated the activity of several cotransfected reporters of the hypertrophic phenotype (atrial natriuretic C peptide, ventricular MLC2, α-skACT2) (Figure 6). Furthermore, cotransfection of a construct expressing either the N17-Ras or
To document increased cell protein content as a marker of hypertrophic growth, we introduced recombinant rUIIR cDNA into neonatal cardiomyocytes using adenoviral vectors, thus introducing recombinant rUIIR into greater than 95% of cultured cells compared with Lipofectamine (typically ~5%). Infection of cardiomyocytes with Ad-rUIIR at an MOI of as little as 12.5 resulted in a modest, yet significant, increase in [\(^{125}\text{I}\)]-UII binding in comparison to noninfected cultures. Moreover, the Ad-rUIIR–infected cells demonstrated a marked hypertrophic growth response to UII (total protein content [\(\mu\text{g/well}\)]; unstimulated, 122.4±4.0; rUII, 147.6±7.0; \(P<0.01\)) equivalent to that observed after stimulation with PE (155.5±6) (Figure 8) with little change in DNA content (\(\mu\text{g/well}\); unstimulated, 3.21±0.3; PE, 3.31±0.3; rUII, 3.16±0.1).

**Discussion**

Myocardial injury is associated with significant structural remodeling, including cardiac hypertrophy and marked interstitial fibrosis.\(^{24}\) Vasconstrictor peptides such as Ang II and ET-1 have emerged as likely candidates\(^{25}\) in these processes. Thus, we sought to determine whether similar actions could be described for the newly identified vasconstrictor peptide UII.

The present study identified the presence of UII and its receptor within myocardial tissue and suggests an important role for UII in cardiac pathophysiology. This is supported by our findings that both UII and its receptor are upregulated after MI in the rat, with these increases distributed to both the infarct and noninfarct zone of the LV. Although this increase in UIIR was observed in all regions measured, it was highest in the right ventricle, consistent with increased pulmonary pressures secondary to LVMI or loss of the myocyte population of cells within the LV, resulting in reduced LV gene expression in this area.

UII peptide was seen in a subpopulation of mast cells. It is of interest that mast cells, identified in larger numbers in both human\(^{26}\) and rat heart\(^{27}\) after ischemic injury, seem to play an important role in the evolution of decompensated cardiac hypertrophy and perivascular fibrosis.\(^{28}\)

The above observations are consistent with those of Douglas et al.,\(^9\) who have reported increased expression of UII and its receptor in the myocardium of patients with congestive heart failure. The findings of the present study, with increases in UII and UIIR observed soon after myocardial injury,
suggest a direct pathophysiological role for the UII system in cardiac remodeling.

An increase in UIIR peptide was observed throughout the myocardium and localized to myocytes, endothelial cells, and fibroblasts, supporting a functional role for UII in cardiac remodeling, acting via these cell types. To explore this potential role for the UII system additionally, we examined the effect of exogenous UII on isolated cardiac cell types in vitro. Analogous to the profibrogenic effects previously documented for other vasoconstrictor peptides, such as Ang II and ET-1, we have found that exogenous UII also stimulates increased matrix synthesis by neonatal cardiac fibroblasts, a key cell type implicated in pathological matrix synthesis within the myocardium. Moreover, this effect was enhanced in the setting of modest UIIR overexpression, suggesting that UII-mediated profibrogenic responses in the post-MI setting could be regulated through altered UIIR expression by resident cardiac fibroblasts and myofibroblasts.

Vasoconstrictor peptides have also been shown to regulate cardiac hypertrophy in response to increased workload, an effect that is mediated predominantly through hypertrophic growth of cardiomyocytes. However, we found that UII does not exhibit an analogous hypertrophic stimulus in quiescent myocytes cultures, as measured by changes in total protein content or in the activation of reporters of the hypertrophic phenotype.

Additional study suggests that the level of rUIIR expression in neonatal cardiomyocytes is below the threshold required for the sustained signaling required for the hypertrophic response. Indeed, in our hands using cultured neonatal cardiomyocytes, even the established hypertrophic hormone Ang II fails to promote hypertrophy; however, infection with an adenovirus encoding the AT1 receptor was necessary to produce a reproducible hypertrophic response to Ang II, strongly implicating receptor upregulation as the defining determinant for initiating hypertrophy. In support of the hypothesis, we found that in cardiomyocytes expressing modest levels of recombinant rUIIR, UII stimulated hypertrophic growth as determined by increased cell protein content as well as activation of phenotypic markers of the hypertrophic response. This effect was mediated, in part, through Gq- and Ras-dependent pathways, analogous to the hypertrophic signaling pathways of other 7-transmembrane-spanning G protein–coupled receptors. Although these observations are made in the in vitro setting (with obvious caution required in their translation into the in vivo setting), these results strongly suggest that, at least in the rat model, the UII system is capable of stimulating cardiac hypertrophy under conditions of receptor upregulation. The potential pathophysiological relevance of this observation is supported by our findings that UIIR gene expression is significantly increased after MI. Similar observations have
been made in established human heart failure.\textsuperscript{9} It is also tempting to speculate that potential upregulation of the UIIR under certain other pathological conditions, such as pressure overload, may contribute to the resultant hypertrophic response. Indeed, upregulation of receptors for other vasoconstrictor peptides has been suggested as a mechanism by which hypertrophic growth is mediated after cardiac injury.\textsuperscript{31}

In conclusion, this study describes, for the first time, upregulation of UII and UIIR in the post-MI setting and profibrogenic effects of UII in isolated cardiac fibroblasts. The present study also suggests that UII may be an important determinant of cardiac hypertrophy in conditions characterized by UIIR upregulation. Moreover, these studies reveal the analogies between UII and other vasoconstrictor peptides has been suggested as a mechanism by which hypertrophic growth is mediated after cardiac injury.\textsuperscript{31}

The present study also suggests that UII may be an important determinant of cardiac hypertrophy in conditions characterized by UIIR upregulation. Moreover, these studies reveal that the intracellular signaling pathways linked to the profibrogenic and hypertrophic effects are likely to be mediated via Gq- and Ras-dependent pathways. Although future studies are required to assess the contribution of endogenous UII on adverse cardiac remodeling in vivo (particularly with the use of specific UIIR antagonists), this report highlights the analogies between UII and other vasoconstrictor peptides implicated in this process, thus supporting a role for UII in the pathogenesis and progression of cardiovascular disease.

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