Direct Inotropic Effects of Exogenous and Endogenous Urotensin-II
Divergent Actions in Failing and Nonfailing Human Myocardium

Michael P. Quaile, PhD; Hajime Kubo, PhD; Carie L. Kimbrough, MPH; Stephen A. Douglas, PhD; Kenneth B. Margulies, MD

Background—Urotensin-II (U-II) is an endogenous peptide upregulated in failing hearts. To date, insights into the myocardial actions of U-II have been obscured by its potent vasoconstrictor effects and interspecies differences in physiological responses to U-II.

Methods and Results—We examined the direct effects of exogenous U-II on in vitro contractility in nonfailing and failing human myocardial trabeculae (n = 47). Rapid cooling contractures (RCC) were used to examine sarcoplasmic reticulum Ca2+ load. In nonfailing myocardium, exogenous U-II increased developed force (DF), rates of force generation and decline and RCC amplitude suggesting increased sarcoplasmic reticulum Ca2+ load. In isolated myocyte suspensions from nonfailing hearts, U-II increased phospholamban phosphorylation. In failing myocardium, exogenous U-II reduced DF and rates of force generation and decline without a significant change in RCC amplitude in trabeculae or a change in phospholamban phosphorylation in myocytes. To examine the effects of endogenous U-II, we administered the peptidic U-II receptor antagonist (UT-A) GSK248451A to isolated trabeculae. UT-A induced a decrease in DF in nonfailing myocardium and an increase in DF in failing myocardium. UT-A increased RCC amplitude slightly in both nonfailing and failing myocardium. During ongoing UT-A, exogenous U-II had little effect on DF and RCC amplitude, confirming effective receptor blockade.

Conclusions—U-II modulates contractility independent of vasoconstriction with opposite effects in failing and nonfailing hearts. Positive inotropic responses to UT-A alone suggests that increased endogenous U-II constrains contractility in failing hearts via an autocrine or paracrine mechanism. These findings support a potential therapeutic role for UT-A in heart failure. (Circ Heart Fail. 2009;2:39-46.)

Key Words: calcium ■ contractility ■ myocardial contraction ■ pharmacology ■ sarcoplasmic reticulum

Numerous studies have demonstrated activation of multiple endogenous neurohormonal systems in the setting of congestive heart failure, and many of these systems have become targets for therapeutic interventions. In this context, recent studies have identified urotensin-II (U-II), a potent endogenous vasoconstrictor peptide, as a potential contributor to the pathogenesis and progression of human heart failure. In addition to the vasculature, U-II and its specific G protein–coupled receptor (UT) are expressed within the heart as well in a variety of other organs (ie, kidneys, adrenal, pancreas, thyroid, and pituitary).

Clinical Perspective see p 46

Circulating plasma levels of U-II are increased in humans with heart failure, and these increases have been inversely correlated with ejection fraction and directly associated with increases in left ventricular filling pressures, dimension, and prognostic markers such as brain natriuretic peptide and endothelin-1. Moreover, several different studies have demonstrated an upregulation of the U-II system (both ligand and receptor) within the failing heart. For example, Douglas et al observed strong expression of the U-II system in the cardiac myocytes and nonmyocytes of failing human hearts compared with lesser expression in the myocardium of patients with mild heart failure and little to no expression in nonfailing hearts.

Although studies have indicated that exogenous U-II can acutely modulate cardiac function, the pathophysiologic implications of increased U-II remain uncertain. Specifically, in vivo vasoconstriction and specie-related differences in myocardial responses to U-II have impeded studies aimed at defining the functional role of U-II in heart failure. For example, in isolated-perfused rat hearts, exogenous U-II...
administration was associated with increases in coronary resistance, negative inotropy, and increases in biomarkers indicative of acute cardiac injury. In this study, detrimental effects of exogenous U-II were even greater in the setting of ischemia-reperfusion injury where upregulation of UT was observed. However, in isolated human right atrial tissue, U-II was found to be a potent isoproterenol with responses implicating a PKC-dependent mechanism and increased phosphorylation of myosin light chain. In ventricular trabeculae obtained from humans, the positive inotropic action of U-II was more potent than endothelin-I or norepinephrine. Interestingly, endothelin-I has known differential effects in failing and nonfailing myocardium, there have been no direct comparisons of myocardial responses to U-II in the presence or absence of heart failure.

Accordingly, we investigated the direct effects of exogenous U-II on in vitro contractile performance in freshly isolated myocardial trabeculae from severely failing human hearts and from nonfailing control hearts. To examine potential autocrine/paracrine effects of endogenous U-II, we also examined the acute myocardial actions of a U-II receptor antagonist (UT-A) on in vitro contractile performance. To provide insight into the mechanisms through which U-II is modulating contractility, we used rapid cooling contractures (RCC) to examine the actions of U-II on sarcoplasmic reticulum (SR) Ca²⁺ load. In addition, we investigated the effects of U-II and UT-A on phospholamban phosphorylation in freshly isolated myocyte suspensions. Our findings indicate that the direct inotropic actions of U-II are qualitatively and quantitatively different in nonfailing and failing human myocardium and support a role for endogenous U-II in the regulation of myocardial contractility.

Methods

Tissue Procurement

Human myocardium was obtained from patients with end-stage heart failure at the time of transplantation and from nonfailing donors whose hearts were deemed unsuitable for transplant. IRB-approved prospective informed consent routines for transplant recipients at the Hospital of the University Pennsylvania, and similar prospective informed consent routines for transplant recipients at the Prussia, Pa.

Table 1. Patient Characterization

<table>
<thead>
<tr>
<th>Group</th>
<th>Age, years</th>
<th>Gender</th>
<th>BW, kg</th>
<th>Etiology</th>
<th>LVEF, %</th>
<th>Medications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonfailing (n=14)</td>
<td>58±4</td>
<td>8 male</td>
<td>78±4</td>
<td>NA</td>
<td>65±6</td>
<td>BB 4/14, ARB 1/14, N 1/14, W 1/14, CCB 1/14</td>
</tr>
</tbody>
</table>

| BW indicates body weight; LVEF: left ventricular ejection fraction; BB, β-blocker; ACEI, ACE inhibitor; ARB, angiotensin receptor blocker; Mil, milrinone; Dig, digoxin; Diu, diuretic; N, nitrates; Amio, amiodarone; Hyd, hydralazine; W, warfarin; CCB, calcium channel blocker. *P<0.05 compared with nonfailing group.
of cells was removed from the cell suspension solution, and the viable cell number was counted using a hemacytometer (Advent Genetics). Cells within the cell suspension solution were centrifuged, the supernatant was discarded, and the cell pellet was resuspended in 1% BSA. Identical aliquots of cells (1.0 mL) were exposed to U-II (0.1, 10, 1000 nmol/L), UT-A (1000 nmol/L) or no drug at all (baseline) for 20 minutes on a shaker (Biotech) at 25°C. Cells were centrifuged for 3 minutes, the BSA supernatant was removed, and the samples were immediately frozen in liquid nitrogen (LN₂) for subsequent molecular analysis.

Western Blot Analysis
Phospholamban levels of phosphorylation were analyzed in isolated myocytes exposed to either U-II or UT-A using Western blot analysis as previously reported. The following antibodies were used: phospholamban (PLB; Upstate) serine-16 and threonine-17 (phosphorylation site specific; PLB; Badrilla).

Statistical Analysis
A Fisher exact test was performed to examine potential differences in subject gender between groups. For continuous variables, results
Cardiomyopathy. Nonfailing hearts were rejected for clinical cardiomyopathy and 10 were from individuals with nonischemic failing hearts, 5 were from individuals with ischemic cardiomyopathy and 10 were from individuals with nonischemic cardiomyopathy. Nonfailing hearts were rejected for clinical transplantation because of chronic hypertension (n=7), donors being over the age of 60 (n=6), or prior viral hepatitis exposure (n=1). The average ejection fraction was 17±2% among failing hearts compared with 65±6% among nonfailing hearts (P<0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>Condition</th>
<th>Dev F, mN/mm²</th>
<th>+dF/dt, mN/s/mm²</th>
<th>TPF, ms</th>
<th>Dia F, mN/mm²</th>
<th>−dF/dt, mN/s/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonfailing (n=13)</td>
<td>Baseline</td>
<td>10.5±0.4</td>
<td>91.0±5.9</td>
<td>191±9.3</td>
<td>3.85±0.5</td>
<td>66.9±5.0</td>
</tr>
<tr>
<td></td>
<td>0.1 nmol/L U-II</td>
<td>14.6±0.7*</td>
<td>121±9.2*</td>
<td>198±9.2</td>
<td>2.85±0.4</td>
<td>97.9±7.5*</td>
</tr>
<tr>
<td></td>
<td>1.0 nmol/L U-II</td>
<td>16.5±1.2*</td>
<td>136±10*</td>
<td>196±9.7</td>
<td>2.94±0.4</td>
<td>102±8.5*</td>
</tr>
<tr>
<td></td>
<td>10 nmol/L U-II</td>
<td>15.0±1.3†</td>
<td>132±12*</td>
<td>188±8.8</td>
<td>2.97±0.3</td>
<td>93.6±8.3†</td>
</tr>
<tr>
<td></td>
<td>100 nmol/L U-II</td>
<td>14.6±1.3†</td>
<td>130±13*</td>
<td>185±9.0</td>
<td>2.63±0.4</td>
<td>88.1±8.5</td>
</tr>
<tr>
<td></td>
<td>1000 nmol/L U-II</td>
<td>14.1±1.2</td>
<td>126±12†</td>
<td>183±15</td>
<td>3.06±0.5</td>
<td>84.6±7.1</td>
</tr>
<tr>
<td>Failing (n=11)</td>
<td>Baseline</td>
<td>17.2±2.4</td>
<td>122±19</td>
<td>235±9.8</td>
<td>4.65±0.7</td>
<td>89.6±18</td>
</tr>
<tr>
<td></td>
<td>0.1 nmol/L U-II</td>
<td>16.3±2.4</td>
<td>128±22</td>
<td>223±13</td>
<td>4.18±0.9</td>
<td>94.2±18</td>
</tr>
<tr>
<td></td>
<td>1.0 nmol/L U-II</td>
<td>14.8±2.0</td>
<td>120±21</td>
<td>224±16</td>
<td>4.53±1.0</td>
<td>79.9±15</td>
</tr>
<tr>
<td></td>
<td>10 nmol/L U-II</td>
<td>13.7±1.7</td>
<td>113±17</td>
<td>224±18</td>
<td>4.49±1.0</td>
<td>76.8±13</td>
</tr>
<tr>
<td></td>
<td>100 nmol/L U-II</td>
<td>12.8±1.5†</td>
<td>109±16</td>
<td>210±12</td>
<td>5.57±1.2</td>
<td>69.0±11</td>
</tr>
<tr>
<td></td>
<td>1000 nmol/L U-II</td>
<td>12.5±1.6†</td>
<td>104±17</td>
<td>216±14</td>
<td>5.93±1.4</td>
<td>60.5±8.5</td>
</tr>
</tbody>
</table>

Responses to U-II receptor antagonism (UT-A) with GSK248451A at 1 μmol/L with and without coadministration of U-II (1 μmol/L) are presented as the mean±SEM. For all hypothesis testing, a probability value of <0.05 was considered to be statistically significant. Differences in the absolute values of all contractile parameters as well as the values obtained through Western blot analysis were analyzed using a 2-way repeated measures ANOVA. When the group by condition interaction was significant, pairwise within-group and between-group comparisons were made using the Hochberg adjustment. Differences in the absolute change from baseline were determined between and within groups using 2 sample and paired t tests, respectively. In these analyses, a Bonferroni adjustment was used to account for multiple pairwise comparisons and assure that values considered statistically significant achieved an experiment-wise probability value of <0.05.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Patient Characteristics

Clinical characteristics of subjects providing myocardial tissue are presented in Table 1. There were no significant differences between age and body weight between patients supplying nonfailing and failing myocardium. Among the failing hearts, 5 were from individuals with ischemic cardiomyopathy and 10 were from individuals with nonischemic cardiomyopathy. Nonfailing hearts were rejected for clinical transplantation because of chronic hypertension (n=7), donors being over the age of 60 (n=6), or prior viral hepatitis exposure (n=1). The average ejection fraction was 17±2% among failing hearts compared with 65±6% among nonfailing hearts (P<0.05).

U-II Concentration-Response

Representative steady-state twitch tracings from U-II concentration-response experiments are shown in Figure 1A and 1B, and absolute values of all contractile parameters are presented in Table 2. U-II induced increases in DF in nonfailing myocardium but decreases in DF in failing myocardium (Figure 1C). In addition, U-II increased the rates of force generation and relaxation (+dF/dt and −dF/dt) in nonfailing myocardium but decreased the rates of force generation and relaxation in failing myocardium (Figure 1D and 1E). Although not precisely defined by these experiments, our findings suggest an EC50 between 0.1 and 1.0 nmol/L for exogenous U-II in human ventricular myocardium. To examine whether intergroup differences in contractility are attributable to U-II–dependent alterations in SR Ca2+ load, RCC experiments were performed. A representative RCC tracing is shown in Figure 2A. In nonfailing myocardium, RCC amplitude increased significantly during graded increases in U-II but remained stable in failing myocardium (Figure 2B).

U-II Receptor Antagonism

Absolute values of all contractile parameters are shown in Table 2 for both nonfailing and failing myocardium before and after bath application of UT-A (1000 nmol/L) alone and on subsequent administration of U-II (1000 nmol/L). UT-A induced a mild decrease in DF in nonfailing myocardium but a pronounced increase in DF in failing myocardium (Figure 3A). UT-A also increased both the rates of force generation...
and relaxation in failing myocardium but had minimal effect on myocardium from nonfailing hearts (Figure 3B and 3C). RCC amplitude increased slightly after UT-A in both failing and nonfailing myocardium (Figure 4). Subsequent administration of U-II during ongoing UT-A had little effect on DF, the rates of force generation and relaxation, and RCC amplitude (Figures 3A through 3C and 4).

Western Blot Analysis
Western blots were performed on freshly isolated human myocyte suspensions to determine the effects of U-II and UT-A on levels of phospholamban phosphorylation. Representative Western blots are shown in Figure 5A for PLB (serine-16 and threonine-17). PLB phosphorylation levels at serine-16 were virtually undetectable in both nonfailing and failing myocytes and were not altered by exposure to U-II or UT-A (Figure 5A). In nonfailing myocytes, U-II induced an inverse dosage-dependent phosphorylation of PLB at threonine-17 above baseline (Figure 5B) and had no effect on failing myocytes (Figure 5C) when the data were normalized by total PLB abundance. In addition, UT-A had no effect on the phosphorylation level of PLB at threonine-17 above baseline (Figure 5B) and had no effect on failing myocytes (Figure 5C) when the data were normalized by total PLB abundance. In addition, UT-A had no effect on PLB phosphorylation at threonine-17 above baseline (Figure 5B) and had no effect on failing myocytes (Figure 5C) when the data were normalized by total PLB abundance. In addition, UT-A had no effect on PLB phosphorylation at threonine-17 above baseline (Figure 5B) and had no effect on failing myocytes (Figure 5C) when the data were normalized by total PLB abundance.

Discussion
The present studies demonstrate striking differences in the direct myocardial actions of exogenous and endogenous U-II in failing and nonfailing human myocardium. By using high quality isolated myocardial preparations under controlled in vitro conditions, our methods permitted fair assessment of pathology-related differences, independent of potentially confounding effects of in vivo vasoconstriction, loading conditions, or other neurohormonal influences. In nonfailing myocardium, exogenous U-II increased DF and the rates of force generation and decline in association with increases in the phosphorylation level of PLB at threonine-17 and RCC amplitude suggesting increases in SR Ca\(^{2+}\) load. However, in failing myocardium, exogenous U-II did not increase DF or the rates of force generation and decline in the absence of changes in PLB phosphorylation or RCC amplitude. Opposite positive inotropic responses to acute UT-A in failing myocardium suggest that endogenous myocardial or vascular U-II is modulating contractility via an autocrine or paracrine effect in the failing heart. These findings indicate modulation of myocardial contractility by endogenous U-II and suggest a potential therapeutic application of UT-A in patients with advanced heart failure.

The syndrome of heart failure is characterized by the chronic activation of several neurohormonal systems. Recent studies have identified the endogenous U-II system as a potential contributor to cardiovascular pathology, including heart failure. Although U-II is present throughout the cardiovascular system and is upregulated in heart failure, its myocardial actions of U-II have been obscured by species-related and disease-related differences. In vivo myocardial responses to U-II may also be influenced by alterations in coronary vascular tone. In this context, our use of isolated myocardial preparations from both failing and nonfailing human hearts to examine the actions of both exogenous and endogenous U-II on in vitro contractile performance, independent of alterations in blood flow, loading conditions, and other neurohormonal influences, is unique among published studies to date.

In nonfailing myocardium, exogenous U-II increased contractility in a concentration-dependent manner. These findings are consistent with previous studies in which U-II increased contractile force generation in isolated human right atrial and ventricular trabeculae. However, in nonfailing ventricular trabeculae, we observed a greater positive inotropic response to U-II than previously reported results derived from a small number of heterogeneous human hearts. Beyond our larger sample size and segregation of failing and nonfailing hearts, our use of perfusion-based cardioplegia and very thin trabeculae without \(\beta\)-adrenergic receptor blocker pretreatment also may have contributed to the higher levels of DF we observed under basal conditions and after U-II exposure. We also observed that U-II increased the rates of force generation and relaxation in nonfailing myocardium. These findings are consistent with increases in the efficiency of intracellular Ca\(^{2+}\) cycling, and the associated increases in the phosphorylation of PLB at threonine-17 and RCCs indicate that SR Ca\(^{2+}\) activity and load is indeed increasing in response to exogenous U-II in nonfailing myocardium. Our observations of an increase in PLB phosphorylation at threonine-17 suggests that the increase in SR Ca\(^{2+}\) load and DF in response to exogenous U-II in nonfailing myocardium is mediated, at least in part, through CaMKII signaling.
In contrast, studies in failing myocardium revealed that exogenous U-II did not increase contractility and exhibited dose-dependent decreases in DF and rates of force generation and relaxation. These divergent myocardial responses to U-II in the presence and absence of heart failure are analogous to the divergent vascular responses to U-II reported by Lim et al in which vasodilation was observed in normal subjects and vasoconstriction was observed in heart failure. The lack of positive inotropic responses to U-II in failing myocardium were associated with an inability to augment RCC amplitude, suggesting an inability to augment Ca\(^{2+}\) cycling or SR Ca\(^{2+}\) load. In this regard, U-II had no effect on the phosphorylation levels of PLB at threonine-17 in failing hearts. Thus, the CaMKII-dependent PLB phosphorylation implicated in the ability of U-II to increase SR load and contractility in the nonfailing heart does not seem to occur with exogenous U-II in the failing heart.

Although additional experiments with skinned fiber preparations did not indicate that U-II affected the force-Ca\(^{2+}\) relationship in nonfailing or failing myocardium, concerns about disruption of UT receptors and downstream signaling by cell permeabilization in these experiments do not permit us to exclude a direct action of U-II on myofilament Ca\(^{2+}\) sensitivity.

Our study indicates that UT-A induces a significant increase in contractility, which is accompanied by an increase in the rates of force generation and relaxation in failing myocardium. Given that these responses were observed in an isolated tissue preparation, our findings suggest that elevated endogenous myocardial U-II exerts a negative inotropic action via an autocrine or paracrine mechanism that is...
independent of changes in tissue perfusion, loading conditions, or circulating neurohormonal mediators in the nonfailing human heart. In addition, positive inotropic responses to UT-A in failing myocardium suggest that the limited response to exogenous U-II may be attributable to previous receptor occupancy. Several factors support the conclusion that responses to UT-A are consequent to inhibition of endogenous tissue U-II activity. First, recent studies demonstrate that GSK248451A is a potent peptidic antagonist with minimal intrinsic activity and high specificity for the U-II receptor. Indeed, in the present study, administration of high-concentration exogenous U-II during ongoing UT-A exposure, induced virtually no physiological changes in both failing and nonfailing myocardium. The fact that contractile responses to UT-A were different in failing and nonfailing myocardium also argues against nonspecific responses. Finally, the observations that contractile responses to UT-A tended to be the inverse of the responses to exogenous U-II in the failing myocardium suggests that UT-A modulated the effects of endogenous U-II within the myocardial tissue.

Overall, the present studies suggest that endogenous U-II is a negative regulator of cardiac contractility in patients with advanced heart failure. This conclusion is based on previous studies demonstrating increases in myocardial U-II expression and content in failing human hearts, our demonstration that exogenous U-II has negative inotropic actions in failing myocardium that are independent of perfusion or loading conditions, and the demonstration that UT-A increases contractility in an isolated tissue model. The positive inotropic effect of UT-A in failing myocardium occurs as a result of an inhibition of endogenous U-II to negatively regulate myocardial contractility. Although the present studies do not address whether the contractility modulating actions of U-II and UT-A will be sustained or attenuated during longer time frames, preclinical data indicate that chronic UT-A treatment reduced adverse cardiac remodeling and lung congestion at 8 weeks after experimental myocardial infarction in rats. Recent studies also implicate U-II in calcineurin-mediated hypertrophic signaling in human hearts. Thus, our findings further support an emerging therapeutic role for UT-A in the treatment of patients with advanced heart failure.

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Disclosures
Drs Quaile and Douglas and Ms Kimbrough currently are or were employees of GlaxoSmithKline.
Effective evidence-based pharmacotherapy for congestive heart failure consists primarily of agents that modulate endogenous vasoactive substances to exert favorable effects on the myocardium or peripheral vasculature. Some agents, like angiotensin antagonists, have little or no direct effects on myocardial systolic performance, yet produce favorable effects on the heart failure syndrome. Although β-adrenergic receptor blockers may induce acute negative inotropic actions necessitating gradual dose titration, they ultimately achieve beneficial effects on myocardial performance. Interestingly, virtually all drugs with acute positive inotropic actions have proven ineffective as disease modifying therapies for heart failure. In this context, the present studies indicate that endogenous urotensin-II (U-II) is a potent vasocostrctor and agonist for the orphan receptor GPR14. Nature. 1999;401:282–286.


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