Exogenous ubiquitin modulates chronic β-adrenergic receptor-stimulated myocardial remodeling: role in Akt activity and matrix metalloproteinase expression

Christopher R. Daniels,* Cerrone R. Foster,* Sana Yakooob, Suman Dalal, William L. Joyner, Mahipal Singh, and Krishna Singh

Department of Biomedical Sciences, James H. Quillen College of Medicine, James H. Quillen Veterans Affairs Medical Center, East Tennessee State University, Johnson City, Tennessee

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Daniels CR, Foster CR, Yakooob S, Dalal S, Joyner WL, Singh M, Singh K. Exogenous ubiquitin modulates chronic β-adrenergic receptor-stimulated myocardial remodeling: role in Akt activity and matrix metalloproteinase expression. Am J Physiol Heart Circ Physiol 303: H1459–H1468, 2012. First published October 5, 2012; doi:10.1152/ajpheart.00401.2012.—β-Adrenergic receptor (β-AR) stimulation increases extracellular ubiquitin (UB) levels, and extracellular UB inhibits β-AR-stimulated apoptosis in adult cardiac myocytes. This study investigates the role of exogenous UB in chronic β-AR-stimulated myocardial remodeling. L-Isoproterenol (ISO; 400 μg·kg⁻¹·h⁻¹) was infused in mice in the presence or absence of UB (1 μg·g⁻¹·h⁻¹). Left ventricular (LV) structural and functional remodeling was studied 7 days after infusion. UB infusion enhanced serum UB levels. In most parts, UB alone had no effect on morphometric or functional parameters. Heart weight-to-body weight ratios were increased to a similar extent in the ISO and UB groups. Echocardiographic analyses showed increased percent fractional shortening, ejection fraction, and LV circumferential stress and fiber-shortening velocity in the ISO group. These parameters were significantly lower in UB vs. ISO. Isovolumic contraction and relaxation times and ejection time were significantly lower in ISO vs. UB + ISO. The increase in the number of TUNEL-positive myocytes and fibrosis was significantly higher in ISO vs. UB + ISO. Activation of Akt was higher, whereas activation of GSK-3β and JNKs was lower in UB + ISO vs. ISO. Expression of MMP-2, MMP-9, and TIMP-2 was higher in UB + ISO vs. ISO. In isolated cardiac fibroblasts, UB enhanced expression of MMP-2 and TIMP-2 in the presence of ISO. Neutralizing UB antibodies negated the effects of UB on MMP-2 expression, whereas recombinant UB enhanced MMP-2 expression. UB activated Akt, and inhibition of Akt inhibited UB + ISO-mediated increases in MMP-2 expression. Thus, exogenous UB plays an important role in β-AR-stimulated myocardial remodeling with effects on LV function, fibrosis, and myocyte apoptosis.

ubiquitin; heart; fibrosis; apoptosis; fibroblasts; myocytes

UBiquitin (UB), a highly conserved protein of ~8.5 kDa, is found in all eukaryotic cells. The most important intracellular function of UB is to regulate protein turnover by the ubiquitin-proteasome pathway (15). The ubiquitin-proteasome pathway may regulate receptor internalization, hypertrophic response, apoptosis, and tolerance to ischemia and reperfusion in cardiac myocytes (56). UB is a normal constituent of plasma. Elevated levels of UB are described in the serum or plasma of patients with parasitic and allergic diseases (4), alcoholic liver disease (48), type 2 diabetes (1), β₂-microglobulin amyloidosis (34), and chronic hemodialysis patients (2). Patients with traumatic brain injury have been shown to have increased UB levels in the cerebrospinal fluid (28). Extracellular UB has been proposed to have pleiotropic functions, including regulation of immune response and anti-inflammatory and neuroprotective activities (27, 29, 36), as well as growth regulation and apoptosis control in hematopoietic cells (9). The biological functions of extracellular UB in the heart remain largely unexplored. Specifically, the role of exogenous UB in myocardial remodeling has not yet been investigated.

Sympathetic nerve activity increases in the heart during cardiac failure. Prolonged stimulation of the β-adrenergic neurohormonal axis contributes to the progression of heart failure and mortality in animal models and human patients (13, 43). Stimulation of β-adrenergic receptor (β-AR) increases expression and activity of matrix metalloproteinase (MMP-2 and MMP-9) in cardiac myocytes in vitro and in vivo (23, 31). It induces apoptosis in cardiac myocytes in vitro and in vivo (19, 42, 43, 55). β-AR-stimulated apoptosis in adult rat ventricular myocytes (ARVMs) is demonstrated to occur via the GSK-3β/JNK-dependent mitochondrial death pathway (30, 39). Recently, we provided evidence that stimulation of β-AR increases extracellular levels of UB in ARVMs, and extracellular UB plays a protective role in β-AR-stimulated apoptosis via the inactivation of GSK-3β and JNK pathways (44).

Here, we investigated the in vivo role of exogenous UB in cardiac myocyte apoptosis and myocardial remodeling following chronic β-AR stimulation in mice. We report that exogenous UB plays an important role in β-AR-stimulated myocardial remodeling with effects on left ventricular function, fibrosis, and myocyte apoptosis. It may inhibit myocyte apoptosis via the activation of Akt and inactivation of GSK-3β and JNKs while also inhibiting fibrosis by modulating expression and activity of MMPs and tissue inhibitors of MMPs (TIMPs).

MATERIALS AND METHODS

Experimental animals. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996). The animal protocol was approved by the East Tennessee State University Committee on Animal Care. Animals were anesthetized using a mixture of isoflurane (2.5%) and oxygen (0.5 l/min), and the heart was excised following a bilateral cut in the diaphragm. Mice were euthanized by exsanguination. The studies were performed using male Institute of Cancer Research (ICR) mice (25–30 g; purchased from Harlan Laboratories).

Mice treatment. Mice were randomly assigned to four groups (sham, ISO, UB + ISO, and UB). The mice in UB + ISO and UB groups received intraperitoneal injection of UB (1 μg/g. U6253;...
**Table 1. Morphometric measurements**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham</th>
<th>ISO</th>
<th>UB + ISO</th>
<th>UB</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>30.27 ± 1.00</td>
<td>31.95 ± 0.86</td>
<td>31.60 ± 0.64</td>
<td>31.00 ± 0.76</td>
<td>NS</td>
</tr>
<tr>
<td>HW, mg</td>
<td>129.7 ± 6.74</td>
<td>167.4 ± 8.98*</td>
<td>178.6 ± 7.46*</td>
<td>144.5 ± 10.8</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>HW/BW, mg/g</td>
<td>4.29 ± 0.21</td>
<td>5.22 ± 0.17*</td>
<td>5.65 ± 0.21*</td>
<td>4.65 ± 0.29</td>
<td>&lt;0.01*</td>
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Values are means ± SE; n = 6. ISO, isoproterenol; UB, ubiquitin; HW, heart weight; BW, body weight; NS, not significant. *Comparison vs. sham.
Echocardiographic measurements. M-mode echocardiography showed no significant difference in LVEDD, LVEDD, %FS, or EF% between the sham and UB groups. ISO infusion decreased LVEDD in ISO but not in the UB + ISO group. LVEDD remained unchanged between the two groups. %FS, EF%, and Vcf were increased in the ISO not in the UB + ISO group compared with sham. In fact, %FS, %EF, and mean Vcf were significantly lower in the UB + ISO compared with the ISO group (Table 2).

Doppler tracings revealed no difference in any parameters between sham and UB groups (Table 3). ISO infusion decreased IVRT, IVCT, and ET in both groups compared with sham. However, the decrease in these parameters was significantly lower in ISO compared with the UB + ISO group (P < 0.05; Table 3). ISO infusion increased heart rates in both groups, with no significant difference between the ISO and UB + ISO groups.

Fibrosis, apoptosis, and hypertrophy. ISO infusion increased fibrosis in both groups (Fig. 2A). Quantitative analysis of trichrome-stained sections showed that the increase in fibrosis was significantly lower in the UB + ISO group compared with ISO (%fibrosis, WT sham: 0.17 ± 0.05; ISO: 1.78 ± 0.15; UB + ISO: 1.09 ± 0.16; UB: 0.29 ± 0.13; P < 0.01 vs. sham, P < 0.05 vs. ISO, n = 5–6; Fig. 2B).

ISO infusion increased the number of TUNEL-positive myocytes in both groups. However, the percentage of apoptotic myocytes was significantly lower in the UB + ISO group compared with ISO (%apoptotic myocyte nuclei/total number of nuclei; sham: 0.07 ± 0.03; ISO: 0.35 ± 0.07; UB + ISO: 0.15 ± 0.05; UB: 0.02 ± 0.01; P < 0.01 vs. sham, P < 0.05 vs ISO, n = 3–5; Fig. 2C).

ISO infusion increased myocyte cross-sectional area to a similar extent in both groups compared with sham (µm²: sham: 137.5 ± 8.5; ISO: 297.4 ± 9.2; UB + ISO: 227.3 ± 28.5; UB: 149 ± 4.8; P < 0.01 vs. sham, n = 3–4; Fig. 2D).

Activation of Akt and GSK-3β. Previously, we provided evidence that inhibition of phosphatidylinositol(PI) 3-kinase inhibits the protective effects of UB in β-AR-stimulated apoptosis in ARVMs (44). PI 3-kinase activates Akt, and activation of Akt plays an antiapoptotic role (22). Western blot analysis of LV lysates using phosophspecific anti-Akt antibodies showed a significant increase in Akt phosphorylation (activity) in both ISO and UB + ISO groups compared with sham. However, the increase in Akt activity was significantly higher in UB + ISO group compared with ISO (P < 0.05; Fig. 3A). UB alone had no effect on Akt activity.

Activation of GSK-3β plays a proapoptotic role in β-AR-stimulated apoptosis (30). Phosphorylation of an NH2-terminal serine residue (Ser9) inactivates GSK-3β. Akt is one of the upstream kinases involved in phosphorylation (Ser9) and inactivation of GSK-3β (16). Western blot analysis of LV lysates using phosphospecific anti-GSK-3β antibodies showed decreased phosphorylation (activation) of GSK-3β in the ISO but not in the UB + ISO group. GSK-3β phosphorylation was significantly higher in the UB + ISO compared with the ISO group (WT sham: 0.76 ± 0.1; ISO: 0.46 ± 0.04; UB + ISO: 0.64 ± 0.04; UB: 0.57 ± 0.1; P < 0.05 vs. sham, P < 0.05 vs. ISO, n = 4–7; Fig. 3B). UB alone had no effect on GSK-3β phosphorylation.

Activation of JNKs and ERK1/2. β-AR-stimulated activation of the JNK pathway is demonstrated to play a proapoptotic role in β-AR-stimulated apoptosis (39). Previously, we provided evidence that active GSK-3β may act upstream in the activation of JNKs (44). Western blot analysis of LV lysates using phosphospecific anti-JNK antibodies showed increased phosphorylation (activation) of JNKs in the ISO but not in the UB + ISO group. Activation of JNKs was significantly lower in UB + ISO group compared with ISO (WT sham: 0.68 ± 0.16; ISO: 4.32 ± 0.35; UB + ISO: 1.88 ± 0.59; UB: 0.99 ± 0.43; P < 0.05 vs. sham, P < 0.05 vs. ISO, n = 4–7; Fig. 4A). UB alone had no effect on JNK phosphorylation. ISO in the presence or absence of UB had no effect on the activation of ERK1/2 (Fig. 4B).

Expression and activation of MMP-2 and MMP-9. MMP-2 and MMP-9 play a significant role in myocardial fibrosis and remodeling (46). Western blot analysis of LV lysates demonstrated increased expression (protein levels) of MMP-2 in both

Table 2. M-mode echocardiographic measurements

<table>
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<tr>
<th>Parameters</th>
<th>Sham (n = 6)</th>
<th>ISO (n = 6)</th>
<th>UB + ISO (n = 6)</th>
<th>UB (n = 5)</th>
<th>Vs. Sham</th>
<th>Vs. ISO group</th>
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<tr>
<td>LVEDD, mm</td>
<td>2.73 ± 0.04</td>
<td>2.36 ± 0.06*</td>
<td>2.78 ± 0.06#</td>
<td>2.74 ± 0.16</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
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<tr>
<td>LVESD, mm</td>
<td>3.90 ± 0.08</td>
<td>3.89 ± 0.02</td>
<td>4.11 ± 0.06</td>
<td>3.97 ± 0.16</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>%FS</td>
<td>30.01 ± 0.99</td>
<td>39.26 ± 1.55*</td>
<td>32.50 ± 1.03#</td>
<td>31.31 ± 1.88</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
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<tr>
<td>EF%</td>
<td>57.73 ± 1.39</td>
<td>70.07 ± 2.02*</td>
<td>61.14 ± 1.45#</td>
<td>59.51 ± 2.84</td>
<td>&lt;0.01</td>
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<td>Mean Vcf, circ/s</td>
<td>5.99 ± 0.23</td>
<td>10.10 ± 0.62*</td>
<td>6.97 ± 0.25#</td>
<td>6.09 ± 0.39</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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Values are means ± SE. LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; %FS, percent fractional shortening; EF%, percent ejection fraction; Vcf, left ventricular circumferential stress and fiber-shortening velocity. *Comparison vs. sham; #comparison vs. ISO.
ISO groups (Fig. 5A). However, the increase in MMP-2 expression was significantly higher in the UB + ISO group compared with ISO. In-gel zymography showed increased MMP-2 activity in both ISO groups. However, the increase in MMP-2 activity was significantly higher in the UB + ISO group compared with ISO (Fig. 5B). Increased MMP-9 expression was observed only in the UB + ISO group (WT sham: 0.79 ± 0.1; ISO: 0.73 ± 0.1; UB: 0.66 ± 0.2; P < 0.05 vs. sham, P < 0.05 vs. ISO, n = 6; Fig. 6A). In-gel zymography failed to show active MMP-9 bands in the LV lysates from these groups (data not shown).

Expression of TIMP-2 and TIMP-4. TIMPs play an important role in regulation of MMP activity (32, 51). Western blot analysis of LV lysates demonstrated that ISO alone has no effect on TIMP-2 protein levels. However, UB alone or in the presence of ISO increased TIMP-2 protein levels significantly (Fig. 6B). ISO in the presence or absence of UB had no effect on protein levels of TIMP-4. UB alone increased TIMP-4 protein levels compared with ISO or UB + ISO groups (data not shown).

UB modulates expression of MMPs and TIMP-2 in cardiac fibroblasts. ISO treatment increased MMP-2 expression in adult cardiac fibroblasts, as analyzed by Western blots. However, the increase in MMP-2 expression was significantly higher in the UB + ISO group compared with ISO (Fig. 7A). In-gel zymography showed a trend toward increased MMP-2 activity in response to ISO. However, MMP-2 activity was significantly higher in the UB + ISO group compared with control and ISO-treated samples (Fig. 7B). These effects of UB on MMP-2 expression were negated by pretreatment with neutralizing anti-UB antibodies (Fig. 7C). Treatment with rUB also enhanced ISO-mediated increases in MMP-2 expression.

### Table 3. Doppler echocardiographic measurements

<table>
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<th>Parameters</th>
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<th>UB (n = 5)</th>
<th>P Value</th>
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<tr>
<td>IVRT, ms</td>
<td>12.41 ± 0.01</td>
<td>5.07 ± 0.01*</td>
<td>7.26 ± 0.01*#</td>
<td>12.98 ± 0.01</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IVCT, ms</td>
<td>11.98 ± 0.01</td>
<td>5.19 ± 0.01*</td>
<td>7.67 ± 0.01*#</td>
<td>13.11 ± 0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ET, ms</td>
<td>50.22 ± 0.01</td>
<td>39.07 ± 0.01*</td>
<td>46.70 ± 0.01*#</td>
<td>51.47 ± 0.01</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>428.37 ± 19.36</td>
<td>572.87 ± 23.7*</td>
<td>506.98 ± 17.98*#</td>
<td>388.64 ± 18.31</td>
<td>&lt;0.05</td>
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Values are means ± SE. IVRT, isovolumetric relaxation time; IVCT, isovolumetric contraction time; ET, ejection time; HR, heart rate. *Comparison vs. sham; #comparison vs. ISO group.

Fig. 2. Myocardial remodeling 7 days after ISO infusion. A and B: analysis of fibrosis. A: Masson’s trichrome-stained sections 7 days after ISO infusion. B: quantitative analysis of fibrosis; n = 5–6. C: quantitative analysis of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling-stained myocytes 7 days after ISO infusion; n = 3–5. D: quantitative analysis of myocyte cross-sectional area; n = 3–4. *P < 0.01 vs. sham; #P < 0.05 vs. ISO. CTL, control.

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However, the effects were observed at a higher concentration (100 μg/ml). MMP-9 expression tended to be higher in the UB + ISO group compared with sham (P = 0.06, n = 6; Fig. 8A). ISO treatment had no effect on TIMP-2 protein levels. However, TIMP-2 protein levels were significantly higher in UB + ISO samples compared with control or ISO-treated samples (Fig. 8B).

**UB activates Akt, and inhibition of Akt inhibits MMP-2 expression in cardiac fibroblast.** To investigate whether UB alone or in combination with ISO activates Akt, cardiac fibroblasts were pretreated with UB followed by treatment with ISO for 15 min. Analysis of cell lysates using phosphospecific antibodies showed that UB or ISO alone had no effect on Akt activation. However, UB in the presence of ISO increased Akt activity significantly compared with control or ISO-treated samples (Fig. 9A). MMP-2 activation is shown to be dependent on activation of Akt in the heart (45). Inhibition of Akt using MK-2206 (18, 37) inhibited UB + ISO-mediated increases significantly in MMP-2 expression (fold change vs. CTL: UB + ISO: 1.71 ± 0.1; MK + UB + ISO: 1.23 ± 0.2; MK: 0.59 ± 0.3; P < 0.05 vs. sham, P < 0.05 vs. UB + ISO, n = 5; Fig. 9B).

**DISCUSSION**

Previously, we provided evidence that β-AR stimulation increases levels of extracellular UB and that treatment with UB plays a protective role in β-AR-stimulated apoptosis in ARVMs (Fig. 7D). However, the effects were observed at a higher concentration (100 μg/ml). MMP-9 expression tended to be higher in the UB + ISO group compared with sham (P = 0.06, n = 6; Fig. 8A). ISO treatment had no effect on TIMP-2 protein levels. However, TIMP-2 protein levels were significantly higher in UB + ISO samples compared with control or ISO-treated samples (Fig. 8B).

**UB activates Akt, and inhibition of Akt inhibits MMP-2 expression in cardiac fibroblast.** To investigate whether UB alone or in combination with ISO activates Akt, cardiac fibroblasts were pretreated with UB followed by treatment with ISO for 15 min. Analysis of cell lysates using phosphospecific antibodies showed that UB or ISO alone had no effect on Akt activation. However, UB in the presence of ISO increased Akt activity significantly compared with control or ISO-treated samples (Fig. 9A). MMP-2 activation is shown to be dependent on activation of Akt in the heart (45). Inhibition of Akt using MK-2206 (18, 37) inhibited UB + ISO-mediated increases significantly in MMP-2 expression (fold change vs. CTL: UB + ISO: 1.71 ± 0.1; MK + UB + ISO: 1.23 ± 0.2; MK: 0.59 ± 0.3; P < 0.05 vs. sham, P < 0.05 vs. UB + ISO, n = 5; Fig. 9B).
This is the first study investigating the role of exogenous UB in the heart specifically in response to chronic β-AR stimulation. Here, we confirm our previous finding of an antiapoptotic function for UB in vivo and show that UB plays an important role in β-AR-stimulated myocardial remodeling with effects on left ventricular function, fibrosis, and myocyte apoptosis. UB infusion enhanced serum UB levels. Exogenous UB depressed β-AR-stimulated increases in systolic and diastolic functional parameters of the heart. Increase in cardiac myocyte apoptosis and myocardial fibrosis was significantly lower in the presence of exogenous UB. Exogenous UB enhanced activation of the antiapoptotic kinase Akt, whereas it decreased the activation of the proapoptotic kinases GSK-3β and JNK. It increased protein levels of MMP-2, MMP-9, and TIMP-2 in the presence of ISO. In isolated cardiac fibroblasts, UB enhanced expression of MMP-2 and TIMP-2. It activated Akt, and inhibition of Akt decreased MMP-2 expression.

Sympathetic nerve activity increases in the heart during cardiac failure. Prolonged stimulation of the β-adrenergic neurohormonal axis contributes to the progression of heart failure and mortality in animal models and human patients (3, 13). Catecholamines, released during heightened adrenergic drive, accumulate in the interstitial space of the heart (6, 8). This accumulation of catecholamines may contribute to left ventricular dysfunction (10). UB is a normal constituent of plasma or serum (11, 29, 47). Levels of UB increase in plasma or serum of patients under a variety of pathological conditions (26). However, the role of plasma UB in the heart has not yet been investigated. Previously, we have shown that β-AR stimulation increases extracellular levels of UB in ARVMs (44). Here, we observed basal presence of UB in the serum of ICR mice. Infusion of UB enhanced serum UB levels in the presence or absence of β-AR stimulation. It was interesting to note that (44). This is the first study investigating the role of exogenous UB in the heart specifically in response to chronic β-AR stimulation. Here, we confirm our previous finding of an antiapoptotic function for UB in vivo and show that UB plays an important role in β-AR-stimulated myocardial remodeling with effects on left ventricular function, fibrosis, and myocyte apoptosis. UB infusion enhanced serum UB levels. Exogenous UB depressed β-AR-stimulated increases in systolic and diastolic functional parameters of the heart. Increase in cardiac myocyte apoptosis and myocardial fibrosis was significantly lower in the presence of exogenous UB. Exogenous UB enhanced activation of the antiapoptotic kinase Akt, whereas it decreased the activation of the proapoptotic kinases GSK-3β and JNK. It increased protein levels of MMP-2, MMP-9, and TIMP-2 in the presence of ISO. In isolated cardiac fibroblasts, UB enhanced expression of MMP-2 and TIMP-2. It activated Akt, and inhibition of Akt decreased MMP-2 expression.
serum UB levels were lower in the presence of β-AR stimulation, and β-AR stimulation alone had no effect on serum UB levels. These observations suggest the possibility that β-AR stimulation may enhance UB absorption in vivo. Isoproterenol is shown to increase cardiac output without affecting renal blood flow in dog, human, or lamb (5, 40). However, the possibility of increased renal blood flow and renal clearance of UB in the presence of isoproterenol cannot be ruled out. A significant finding of this study is that exogenous UB has the potential to play an important role in the remodeling process of the heart by affecting β-AR-stimulated increases in myocyte apoptosis and myocardial fibrosis.

Ventricular hypertrophy is considered to be an important compensatory mechanism that allows the heart to maintain its output. Chronic β-AR stimulation is shown to induce hypertrophy and increase heart rate and LV systolic function (20, 49, 50). Indicators of hypertrophy such as HW/BW ratio and myocyte cross-sectional area were increased to a similar extent in both ISO groups. An interesting finding of this study is that exogenous UB in the presence of ISO restored systolic function to normal levels, as indicated by decreased %FS, %EF, and Vcf. It partially restored ISO-mediated decrease in IVRT, IVCT, and ET. Changes in heart rate are suggested to affect echocardiographic parameters in mice (52). As observed previously (23), ISO infusion increased heart rate in ICR mice. UB alone or in the presence of ISO had no effect on basal or ISO-mediated increases in heart rates. Therefore, the observed changes in echocardiographic parameters between ISO and UB ISO groups may not be due to the changes in heart rate. Extracellular UB is shown to promote intracellular Ca++ flux and reduce cAMP levels through a G protein-coupled receptor in THP1 cells (41). Therefore, UB may affect systolic and...

Fig. 7. Expression and activity of MMP-2 in cardiac fibroblasts. A and B: cardiac fibroblasts were pretreated with UB (10 μg/ml) for 30 min, followed by treatment with ISO (10 μM) for 48 h. A: cell lysates (20 μg) were analyzed by Western blot using anti-MMP-2 antibodies. *P < 0.05 vs. CTL; #P < 0.05 vs. ISO; n = 11. B: concentrated conditioned media (2 μg) were analyzed by gelatin in-gel zymography. *P < 0.01 vs. CTL; #P < 0.05 vs. ISO; n = 3. C: cells were pretreated with neutralizing anti-UB antibody (Ab) for 30 min, followed by treatment with UB for 30 min. The cells were then treated with ISO for 48 h. Cell lysates (20 μg) were analyzed by Western blot using anti-MMP-2 antibodies. *P < 0.05 vs. CTL; $P < 0.05 vs. UB; n = 3. D: cells were pretreated with recombinant UB (rUB; 10 or 100 μg/ml) for 30 min, followed by treatment with ISO for 48 h. Cell lysates (20 μg) were analyzed by Western blot using anti-MMP-2 antibodies. *P < 0.05 vs. CTL; #P < 0.05 vs. ISO; n = 3. Actin immunostaining indicates protein loading.
that activation of the PI 3-kinase/Akt pathway may be a mechanism involved in antiapoptotic effects of exogenous UB.

Chronic sympathetic stimulation is shown to induce growth of interstitial tissue in the heart, leading to fibrosis (7). MMPs and TIMPs play an important role in the remodeling of extracellular matrix (46). Previously, we have shown that chronic β-AR stimulation increases myocardial fibrosis (23). Consistent with these observations, we observed increased fibrosis following chronic β-AR stimulation in ICR mice. The new finding of this study is that exogenous UB inhibits chronic β-AR-stimulated increases in myocardial fibrosis. It enhanced
diastolic parameters of the heart by modulating levels of intracellular Ca²⁺ and/or cAMP. However, further investigations are needed to understand the mechanism by which exogenous UB modulates heart function in the presence of an β-AR agonist.

Cardiac myocyte apoptosis plays a crucial role in the pathogenesis of heart failure (17, 21, 33). β-AR-stimulated activation of JNKs and GSK-3β plays a proapoptotic role via the involvement of mitochondrial death pathway (30, 39). Previously, UB has been shown to activate PI 3-kinase and inhibit β-AR-stimulated activation of JNKs and GSK-3β and mitochondrial death pathway of apoptosis (44). PI 3-kinase is an upstream activator of Akt (35), whereas Akt acts upstream in the inactivation of GSK-3β (16). Active GSK-3β may act upstream in the activation of JNKs (44). Here, we show that exogenous UB enhances β-AR-stimulated activation of Akt, whereas it inhibits activation of GSK-3β and JNKs. These in vivo data confirm our previous in vitro findings and suggest

Fig. 8. Expression of MMP-9 and TIMP-2. Cardiac fibroblasts were pretreated with UB (10 μg/ml) for 30 min, followed by treatment with ISO (10 μM) for 48 h. Cell lysates (20 μg) were analyzed by Western blot using anti-MMP-9 or anti-TIMP-2 antibodies. Actin immunostaining indicates protein loading. A: expression of MMP-9. #P = 0.06 vs. ISO; n = 6. B: expression of TIMP-2. *P < 0.05 vs. CTL; #P < 0.05 vs. ISO; n = 3–4.

Fig. 9. Activation of Akt and its role in MMP-2 expression. A: cardiac fibroblasts were pretreated with UB (10 μg/ml) for 30 min, followed by treatment with ISO (10 μM) for 15 min to measure Akt activation. B: to measure MMP-2 expression, cardiac fibroblasts were pretreated with MK-2206 (MK; 1 μM) for 30 min and then with UB (10 μg/ml) for 30 min, followed by treatment with ISO (10 μM) for 48 h. Cell lysates (20 μg) were analyzed by Western blot using phosphospecific anti-Akt (Ser473) or anti-MMP-2 antibodies. A and B, bottom, exhibit mean data normalized to total Akt or actin. A: phosphorylation (activation) of Akt. *P < 0.05 vs. CTL; #P < 0.05 vs. ISO; n = 5. B: expression of MMP-2. *P < 0.05 vs. CTL; #P < 0.05 vs. UB + ISO; n = 5.
chronic β-AR-stimulated increases in the expression and activity of MMP-2. It also increased expression of MMP-9 and TIMP-2 in the heart. Use of isolated adult cardiac fibroblasts confirmed our in vivo findings with respect to the expression of MMPs and TIMP-2 in response to UB. Of note, a higher concentration of rUB (compared with bovine UB) was required to observe increased expression of MMP-2 in the presence of isoproterenol in fibroblasts (Fig. 7D). The reasons may include differential modification of UB from different sources. UB is shown to be modified by acetylation of lysines, oxidation of methionine, and nitration of tyrosine (25, 54). These modifications may influence interaction of extracellular UB with its receptor and/or other proteins. The concentration of TIMP-2 is suggested to determine the role of TIMP-2 in activation of MMP-2. At low concentrations, TIMP-2 may activate MMP-2 on the cell surface with the MT1/MMP/TIMP-2 complex serving as a receptor for proMMP-2, whereas at higher concentrations TIMP-2 may neutralize MT1/MMP and prevent activation of MMP-2 (24). The data presented here suggest that exogenous UB decreases chronic β-AR-stimulated myocardial fibrosis by modulating expression of MMPs and TIMP-2.

Previously, we have reported that chronic β-AR stimulation increases MMP-9 expression in the heart (23). Here, we did not observe increased expression of MMP-9 in response to chronic β-AR stimulation. The observed discrepant findings may relate to the use of different mouse strains. A previous study used 129xblack Swiss mice, whereas the current study uses ICR mice (23).

Increased MMP-2 expression is suggested to be dependent on Akt activation in the rat heart during ischemia-reperfusion (45). In the current study, UB enhanced Akt activation in the heart and in isolated cardiac fibroblasts in the presence of β-AR stimulation. Inhibition of Akt decreased UB + ISO-mediated increases in MMP-2 expression. These data suggest a potential relationship between Akt activation in response to UB treatment and MMP-2 expression in the heart.

Perspective. The data presented here are novel and of significant interest since exogenous UB modulates β-AR-stimulated myocardial function and inhibits myocardial fibrosis and cardiac myocyte apoptosis. The structural changes related to cardiac myocyte apoptosis and extracellular matrix play a significant role in modulation of myocardial function and in the progression to heart failure. Therefore, elucidation of processes that can shift the balance from myocyte apoptosis to survival may have clinical implications. In addition, analysis of components of extracellular matrix, including collagen type I and IV, laminin, fibronectin, etc., may provide insight into the modulation of heart function in the presence of exogenous UB. It should be emphasized that our data on heart function and signaling are obtained 7 days after ISO infusion. The experimental time point should be extended beyond 7 days to investigate long-term effects of exogenous UB.

REFERENCES


