Urocortin protects the heart from reperfusion injury via upregulation of p42/p44 MAPK signaling pathway

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Schulman, Daniel, David S. Latchman, and Derek M. Yellon. Urocortin protects the heart from reperfusion injury via upregulation of p42/p44 MAPK signaling pathway. Am J Physiol Heart Circ Physiol 283: H1481–H1488, 2002. First published June 13, 2002; 10.1152/ajpheart.01089.2001.—Reperfusion of ischemic myocardium is essential for tissue salvage but paradoxically contributes to cell death. We hypothesized that activation of potential survival pathways such as p42/p44 MAPK may prevent lethal reperfusion injury. Urocortin is a peptide factor that affects the p42/p44 MAPK signaling pathway. Both isolated and in vivo rat heart models were used to examine the potential for urocortin to prevent reperfusion injury. Isolated rat hearts underwent 35-min regional ischemia and 2-h reperfusion, with urocortin perfused for 20 min from the onset of reperfusion. In the in vivo study, urocortin was administered as an intravenous bolus 3 min before reperfusion with a protocol of 25-min regional ischemia and 2-h reperfusion. Blockade of the p42/p44 MAPK pathway with the inhibitor PD-98059 was used in both models. Urocortin attenuated lethal reperfusion-induced injury both in vitro and in vivo via a p42/p44 MAPK-dependent mechanism. Furthermore, Western blot analysis demonstrated the ability of urocortin to directly upregulate this signaling pathway. In conclusion, we believe that the p42/p44 MAPK-dependent signaling pathway represents an important survival mechanism against reperfusion injury. Isolated rat hearts underwent 35-min regional ischemia and 2-h reperfusion. Blockade of the p42/p44 MAPK pathway with the inhibitor PD-98059 was used in both models. Urocortin attenuated lethal reperfusion-induced injury both in vitro and in vivo via a p42/p44 MAPK-dependent mechanism. Furthermore, Western blot analysis demonstrated the ability of urocortin to directly upregulate this signaling pathway. In conclusion, we believe that the p42/p44 MAPK-dependent signaling pathway represents an important survival mechanism against reperfusion injury.

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METHODS

The investigation conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996).

**Isolated rat heart preparation.** After pentobarbital sodium (50 mg/kg ip) anesthesia was administered, the hearts of 40 male Sprague-Dawley rats (280–350 g) were excised and retrogradely perfused on a Langendorff apparatus (at 80 mmHg). Temperature was maintained at 37 ± 0.5°C and a water-filled latex balloon was inserted into the left ventricle and inflated to set end-diastolic pressure at 5–10 mmHg. Regional ischemia was achieved by pulling on a snare passed around the main branch of the left coronary artery and confirmed by a substantial fall in both left ventricular developed pressure (LVDP) and coronary flow (CF). All hearts underwent 15 min of stabilization, 35 min of regional ischemia, and 2 h of reperfusion. Procedural exclusion criteria included a rate pressure product (RPP) <18,000 mmHg·beat⁻¹·min at the end of the stabilization period, an inability to maintain >80% of initial contractile function through the stabilization period, a CP <8 and >16 ml·min⁻¹·100 g tissue⁻¹ at the end of the stabilization, and persistent arrhythmias throughout stabilization. Four groups were included in this study (Fig. 1A). Control hearts underwent 35 min of regional ischemia and 2 h of reperfusion. Further groups had urocortin (10⁻⁸ mol/l) alone or both urocortin (10⁻⁸ mol/l) and PD-98059 (5 μmol/l) or PD-98059 (5 μmol/l) alone perfused for the first 20 min of reperfusion starting 3 min before the onset of reperfusion.

**Experimental groups for phosphorylated p42/p44 MAPK assessment.** Various experimental groups (n = 3–5 per group) were studied for analysis of phospho-p42/p44 MAPK using the isolated perfused rat heart. Sham hearts were used after a 60-min perfusion (stabilization). Control experiments followed the same protocol as the infarct studies: 35-min regional ischemia and 2-h reperfusion. Individual hearts were biopsied once from the cardiac apex after 32-min ische-

![Fig. 1. A: protocol for the in vitro isolated perfused rat heart study. The experimental groups include: control, urocortin (10⁻⁸ M), urocortin + PD-98059, and PD-98059 (5 μM). B: protocol for the in vivo rat heart study. Experimental groups include: control, urocortin (1 μg/kg), urocortin (15 μg/kg), PD-98059 (4 mg/kg), urocortin (15 μg/kg) + PD-98059 (4 mg/kg), and glyceryl trinitrate (GTN).](http://ajpheart.physiology.org/)

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mia or after 35-min ischemia, followed by 5-, 10-, 20-, and 120-min reperfusion. Hearts perfused with urocortin at reperfusion after 35-min ischemia were biopsied after 5-, 10-, 20-, and 120-min reperfusion. The hearts treated with both urocortin and PD-98059 or PD-98059 alone were biopsied after 35-min ischemia and 10-min reperfusion. All drugs were perfused following the same protocols used for the in vitro infarct-based studies. At the end of each experiment the estimated risk zone at the apex of the heart was rapidly frozen in liquid nitrogen and used later for Western blot analysis of the phosphorylated form of p42/p44 MAPK with the use of computerized planimetry program (Summa Sketch II, Summa Graphics; Seymour, CT), the respective volumes were calculated. Infarct size was expressed as a percentage of the risk zone. All measurements were performed in a blinded fashion.

**Statistical analysis.** Data from the experiments are expressed as means ± SE. Differences between the means were compared using the Student’s t-test. The risk and infarct volumes were tested for group differences by a one-way ANOVA. Comparisons of CF (normalized to the heart weight and expressed as ml·min⁻¹·100 g tissue⁻¹), RPP (calculated from HR × LVDP), and BP were performed by repeated measures of ANOVA. *P < 0.05 was considered significant.

**Drugs and materials.** Urocortin and PD-98059 were obtained from Sigma (Dorset, UK). Urocortin was initially dissolved in a 0.01% acetic acid solution and PD-98059 was dissolved in dimethyl sulfoxide (DMSO) with a final in vitro concentration of 0.02%. PD-98059 was injected intravenously, dissolved in 0.2 ml 99.5% DMSO solution, and the cannula was subsequently flushed with 0.09% saline. Neither vehicle had any inherent effect on infarct size. GTN (1 mg/ml) was obtained from Faulding (Leamington Spa, UK) and diluted to the final concentration in 0.09% saline.

**RESULTS**

Urocortin protects in vitro isolated perfused heart from reperfusion injury via p42/p44 MAPK pathway. Urocortin (10⁻⁸ M) perfused for the first 20 min of reperfusion significantly reduced infarct size from 49.3 ± 2.2% in control hearts to 22.7 ± 2.9% in urocortin-treated hearts (*P < 0.01) (Fig. 2). The MEK1 inhibitor PD-98059 completely abrogated the protective effects of urocortin with infarct sizes returning to 49.8 ±

![Image](http://ajpheart.physiology.org/...).
Urocortin protects heart from reperfusion injury in anesthetized in vivo adult rat via p42/p44-dependent signaling pathway. To advance the clinical relevance of the study, we examined for the first time the cardioprotective effect of urocortin using the in vivo rat heart model. Urocortin administered just before reperfusion was also seen to significantly reduce myocardial infarction in a dose-dependent manner (Fig. 5). Infarct size was reduced from 48.6 ± 2.6% in control hearts to 29.3 ± 2.7% in rats treated with 15 µg/kg urocortin (P < 0.01). Rats treated with 1 µg/kg of urocortin at reperfusion showed no reduction in infarct size (54.8 ± 4.5%) compared with control hearts. When PD-98059 (4 mg/kg) was injected intravenously 3 min before urocortin (15 µg/kg), the infarct reduction was abolished and infarct sizes were comparable to control hearts (47.0 ± 4.3 vs. 48.6 ± 2.6%). PD-98059 (4 mg/kg) alone had no effect on infarct size compared with control hearts (55.4 ± 5.7 vs. 48.6 ± 2.6%).

Mean arterial BP, HR, and arterial blood gas analysis for the in vivo study. Mean arterial pressure and HR measurements (Table 3) were recorded throughout the ischemia-reperfusion protocol. Both urocortin at 1 and 15 µg/kg significantly reduced the mean arterial pressure (P < 0.05) compared with control after intravenous infusion, and this reduction was maintained throughout the reperfusion period. Only at 5- and 60-min reperfusion was the mean arterial pressure of the group treated with urocortin 15 µg/kg significantly less than that treated with urocortin 1 µg/kg (P < 0.05). In the urocortin 15 µg/kg-treated group, a significantly higher HR was noted at 60 and 120 min of reperfusion. GTN was infused intravenously at a variable rate (and not continuously) to mimic the hypotensive effects of urocortin 15 µg/kg. No significant changes in HR were noted in this group compared with control. DMSO, the vehicle in which PD-98059 was solubilized, caused a transient hypertensive effect that normalized within 5 min reperfusion.

Table 2. CFR at baseline, body weight, heart weight, heart weight/body weight ratio, and risk zone analysis for all in vitro groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>CFR Baseline, ml·min⁻¹·100 g⁻¹</th>
<th>BW, g</th>
<th>HW, g</th>
<th>HW/BW Ratio</th>
<th>Risk Zone, cm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.5 ± 0.0</td>
<td>340 ± 16</td>
<td>1.3 ± 0.0</td>
<td>3.8 ± 0.3</td>
<td>0.64 ± 0.03</td>
</tr>
<tr>
<td>Urocortin</td>
<td>11.6 ± 0.5</td>
<td>334 ± 8</td>
<td>1.3 ± 0.0</td>
<td>3.9 ± 0.1</td>
<td>0.54 ± 0.04</td>
</tr>
<tr>
<td>Urocortin + PD-98059</td>
<td>11.6 ± 0.5</td>
<td>325 ± 15</td>
<td>1.2 ± 0.1</td>
<td>3.7 ± 0.2</td>
<td>0.66 ± 0.06</td>
</tr>
<tr>
<td>PD-98059</td>
<td>11.2 ± 0.3</td>
<td>328 ± 11</td>
<td>1.3 ± 0.0</td>
<td>3.9 ± 0.1</td>
<td>0.65 ± 0.03</td>
</tr>
</tbody>
</table>

Values are means ± SE. CFR, coronary flow rate; BW, body weight; HW, heart weight.
This had no effect on infarct size. The arterial pH was maintained between 7.35 and 7.45 for the duration of the experiments, and the PO2 was held between 25 and 35 kPa. The whole body and heart weights and the risk zones were similar for all groups studied (Table 4).

**Hypotensive effect of GTN does not affect infarct size.**

To ascertain whether the hypotensive effect seen with urocortin could possibly account for the protection observed, we examined the effect of GTN used intermittently at a dose that mirrored the hypotension caused by 15 μg/kg urocortin at reperfusion. This hypotensive effect of GTN did not cause any reduction in infarct size (Fig. 5). The infarct size of the GTN-treated group was no different from that of control hearts (48.8 ± 4.2 vs. 48.6 ± 2.6%). Therefore, the specific cardioprotective of urocortin was unrelated to hypotension.

**DISCUSSION**

Reperfusion remains the only means of salvaging ischemic myocardium and limiting infarct development. The challenge of developing a reliable means of protecting the heart from reperfusion-induced injury must be met if we are to beneficially affect the survival of patients suffering from myocardial infarction. We show that urocortin, acting via a p42/p44 MAPK-dependent signaling pathway, is able to protect both the in vitro and in vivo rat heart from reperfusion injury possibly through direct upregulation of a reperfusion injury salvage kinase.

Although there are inherent limitations when in vitro systems attempt to simulate in vivo ischemia and reperfusion, cell-based studies have shown that simulated reperfusion (or reoxygenation) results not in the accelerated lysis of already dead cells, but rather in lethal injury to cells that were previously viable (25). The ischemic alterations of cellular conditions are however, necessary prerequisites and indeed set the stage for lethal reperfusion injury.

In neonatal cardiomyocytes, Brar et al. (3) showed that urocortin can significantly attenuate the injury of reoxygenation as measured using markers of both necrosis and apoptosis. This protection was shown to be independent of urocortin-induced p38 MAPK or JNK phosphorylation, and specific inhibition of p38 MAPK failed to inhibit the cardioprotective effect of urocortin (3). In this study, we have demonstrated in the intact heart (both in vitro and in vivo), the importance of urocortin acting via the p42/p44 MAPK in protecting the heart against infarct-related cardiac dysfunction. MAPKs are an important area of focus to elucidate the complex relationship between signal transduction and this balance of survival and death in the ischemic myocardium. The dynamic relationship of their activities may be important in determining the outcome of the cardiomyocyte after the stress of reperfusion. In our study, we have demonstrated the ability of urocortin to augment the phosphorylation of p42/p44 MAPK in the reperfused myocardium and suggest that it is...
possibly through this mechanism that the myocardium is protected from lethal reperfusion injury. However, it is possible that the increased level of infarction in control hearts directly reduced detectable levels of phospho p42/p44 MAPK in these tissues. It is also possible that the rapid decline in phospho-p42/p44 MAPK after 10-min reperfusion despite the presence of urocortin may be the result of MAPK phosphatase induction, which has previously been demonstrated to cause dephosphorylation and inactivation of p42/p44 MAPK (8). It should be emphasised that in our study the effect of PD-98059 was effective in both attenuating the protection (infarct size) obtained with urocortin at reperfusion and attenuating the phosphorylation and downregulation of the p42/p44 MAPK.

It is not fully understood how p42/p44 MAPK up-regulation may protect the myocardium against the consequences of reperfusion injury. p42/p44 MAPK and BMK1 have been shown to protect against ischaemia-reperfusion-induced cardiomyocyte cell death (21, 22). In addition to the above study, one could speculate that the p90 ribosomal S6 kinase may have special functions as a substrate of the p42/p44 MAPK (7), both by inhibiting components of the cell death machinery [e.g., phosphorylation and inhibition of Bcl-2 associated death promoter (BAD)] (23) and increasing transcription of prosurvival genes. p42/p44 MAPK acting downstream of B-Raf may also inhibit cytosolic caspase activation following release of cytochrome c from the mitochondria (6). Furthermore, protein kinase B (PKB) has been shown to inactivate caspase-9 through phos-

![Image 44x439 to 296x724]

**Fig. 4.** The effect of urocortin on p42/p44 MAPK phosphorylation in the in vitro isolated heart after 35-min ischemia and 10-min reperfusion is abrogated by PD-98059. Experimental groups include a comparison of untreated hearts vs. groups treated with urocortin or urocortin + PD-98059 or PD-98059 alone. *P < 0.05 compared with time-matched control.

![Image 235x58 to 559x366]

**Fig. 5.** Urocortin (15 μg/kg) administered at reperfusion in vivo reduces infarct size via p42/p44 MAPK pathway. *P < 0.01.
phosphorylation (4), though other serine-threonine kinases may also modify such caspase activation. It seems that p42/p44 MAPK and PKB (4) are both able to target BAD and inactivate its proapoptotic function through phosphorylation at two different sites (protein kinase A is able to inactivate BAD through a third phosphorylation site) (9). PKB has also been shown to maintain mitochondrial membrane integrity and prevent release of cytochrome c independent of BAD phosphorylation (14). This effect may be partly explained by the ability of PKB to activate mitochondrial Raf-1 and the downstream p42/p44 MAPK pathway (16). It should be noted that although we believe urocortin capable of protecting against apoptosis in the heart, our end point in the whole heart has been that of infarct size reduction.

In our study, the hemodynamic effects of urocortin in vivo appeared to play no role in the cardioprotection from reperfusion injury. Although a significant reduction in mean arterial pressure was found in the group treated with 1 μg/kg urocortin, no cardioprotective effect was seen. Hypotension during reperfusion has not been shown to limit infarct size. To examine this in more detail we used intravenous GTN intermittently during reperfusion to mimic the hemodynamic effect of 15 μg/kg urocortin as closely as possible. Although we were able to significantly reduce the mean arterial pressure comparable to 15 μg/kg urocortin, no reduction of infarct size was noted. Whereas GTN (and other nitrates) have been shown to protect the myocardium if administered before ischemia (11), these agents have not been shown to protect the heart from lethal reperfusion injury (when given at reperfusion). Furthermore, we saw no changes in cardiac function in the in vitro experiments, where urocortin maintained a powerful protective effect in a neutrophil free crystallloid buffer-perfused Langendorff system.

Studies (20) in chronically instrumented conscious sheep have showed that urocortin induces a dose-dependent increase in HR, cardiac output, mean arterial pressure, and CRF rate (doses of between 1 and 100 μg injected intravenously). There was no change in peripheral vascular conductance and stroke volume. All urocortin-induced cardiovascular effects were inhibited by prior treatment with the CRF antagonist α-helical CRF. In contrast, however, when urocortin was administered in freely moving rats it produced a pronounced and prolonged reduction of mean arterial pressure (as demonstrated in our anesthetized rats). Both the size and duration of this effect were dose dependent (26). These disparate hemodynamic actions of urocortin in the sheep and rat may be due to species-specific effects of urocortin on the sheep and rat, possibly due to the different CRF receptors.

Table 3. MAP and HR at regular time intervals throughout the ischemia-reperfusion in vivo protocol

<table>
<thead>
<tr>
<th>Groups</th>
<th>Preischemia</th>
<th>5-Min Ischemia</th>
<th>25-Min Ischemia</th>
<th>5-Min Reperfusion</th>
<th>60-Min Reperfusion</th>
<th>120-Min Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>121 ± 7</td>
<td>109 ± 6</td>
<td>101 ± 7</td>
<td>99 ± 4</td>
<td>97 ± 7</td>
<td>86 ± 8</td>
</tr>
<tr>
<td>HR</td>
<td>457 ± 13</td>
<td>463 ± 9</td>
<td>464 ± 12</td>
<td>443 ± 12</td>
<td>437 ± 10</td>
<td>437 ± 9</td>
</tr>
<tr>
<td>Urocortin (1 μg/kg)</td>
<td>124 ± 5</td>
<td>110 ± 4</td>
<td>88 ± 7</td>
<td>73 ± 7*</td>
<td>70 ± 7*</td>
<td>59 ± 5*</td>
</tr>
<tr>
<td>HR</td>
<td>470 ± 6</td>
<td>465 ± 10</td>
<td>467 ± 16</td>
<td>465 ± 10</td>
<td>455 ± 12</td>
<td>453 ± 12</td>
</tr>
<tr>
<td>Urocortin (15 μg/kg)</td>
<td>121 ± 6</td>
<td>106 ± 6</td>
<td>70 ± 6*</td>
<td>54 ± 3*</td>
<td>50 ± 3*</td>
<td>50 ± 2*</td>
</tr>
<tr>
<td>HR</td>
<td>453 ± 10</td>
<td>485 ± 10</td>
<td>472 ± 13</td>
<td>466 ± 11</td>
<td>483 ± 9*</td>
<td>466 ± 11*</td>
</tr>
<tr>
<td>PD-98059 (4 mg/kg)</td>
<td>125 ± 8</td>
<td>115 ± 5</td>
<td>128 ± 8*</td>
<td>97 ± 7</td>
<td>96 ± 9</td>
<td>93 ± 4</td>
</tr>
<tr>
<td>MAP</td>
<td>470 ± 10</td>
<td>485 ± 10</td>
<td>480 ± 14</td>
<td>477 ± 12</td>
<td>450 ± 17</td>
<td>455 ± 15</td>
</tr>
<tr>
<td>HR</td>
<td>117 ± 6</td>
<td>108 ± 9</td>
<td>111 ± 8</td>
<td>69 ± 5*</td>
<td>61 ± 2*</td>
<td>62 ± 5*</td>
</tr>
<tr>
<td>Urocortin (15 μg/kg)/PD-98059 (4 mg/kg)</td>
<td>440 ± 12</td>
<td>440 ± 14</td>
<td>443 ± 11</td>
<td>437 ± 15</td>
<td>420 ± 13</td>
<td>423 ± 7</td>
</tr>
<tr>
<td>MAP</td>
<td>115 ± 9</td>
<td>109 ± 8</td>
<td>66 ± 3*</td>
<td>53 ± 2*</td>
<td>48 ± 3*</td>
<td>49 ± 2*</td>
</tr>
<tr>
<td>HR</td>
<td>450 ± 13</td>
<td>468 ± 15</td>
<td>466 ± 15</td>
<td>444 ± 15</td>
<td>426 ± 14</td>
<td>438 ± 12</td>
</tr>
</tbody>
</table>

Values are means ± SE. MAP, mean arterial pressure; HR, heart rate; GTN, glyceryl trinitrate. *P < 0.005, compared with control.

Table 4. Body weight, heart weight, heart weight/body weight ratio, and risk zone analysis for all in vivo groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>BW, g</th>
<th>HW, g</th>
<th>HW/BW</th>
<th>Risk Zone, cm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>349 ± 11</td>
<td>1.2 ± 0.02</td>
<td>3.5 ± 0.13</td>
<td>0.59 ± 0.03</td>
</tr>
<tr>
<td>Urocortin (1 μg/kg)</td>
<td>339 ± 6</td>
<td>1.2 ± 0.05</td>
<td>3.6 ± 0.20</td>
<td>0.56 ± 0.03</td>
</tr>
<tr>
<td>Urocortin (15 μg/kg)</td>
<td>351 ± 8</td>
<td>1.2 ± 0.04</td>
<td>3.4 ± 0.09</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>PD-98059 (2 mg/kg)</td>
<td>358 ± 7</td>
<td>1.2 ± 0.04</td>
<td>3.4 ± 0.11</td>
<td>0.52 ± 0.03</td>
</tr>
<tr>
<td>PD-98059 (4 mg/kg)</td>
<td>346 ± 11</td>
<td>1.2 ± 0.07</td>
<td>3.5 ± 0.26</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>Urocortin (15 μg/kg)/PD-98059 (2 mg/kg)</td>
<td>342 ± 17</td>
<td>1.2 ± 0.02</td>
<td>3.4 ± 0.15</td>
<td>0.50 ± 0.04</td>
</tr>
<tr>
<td>Urocortin (15 μg/kg)/PD 4 mg/kg)</td>
<td>364 ± 15</td>
<td>1.3 ± 0.02</td>
<td>3.6 ± 0.15</td>
<td>0.54 ± 0.06</td>
</tr>
<tr>
<td>GTN</td>
<td>345 ± 7</td>
<td>1.2 ± 0.02</td>
<td>3.5 ± 0.08</td>
<td>0.50 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SE.
specific differences in the binding of CRF and urocortin to peripheral CRF-R2. Mice generated lacking expression of CRF-R2 have been shown to have higher resting BPs, and in response to systemic urocortin fail to show any enhanced cardiac performance or reduced BP (5).

In conclusion, we have shown for the first time that urocortin can protect the intact heart from reperfusion injury both in vitro and in vivo and that this protection appears to be associated with upregulation of a p42/p44 MAPK-dependent signaling pathway. Future studies will need to investigate the importance of the p42/p44 MAPK pathways in mediating the protective effect of both urocortin as well as other potential therapies that may affect this signaling pathway. We believe the heart possesses pro-survival reperfusion injury salvage kinases pathways that may be exploited when developing agents that can be used to protect the myocardium against the consequences of lethal reperfusion injury.

REFERENCES


