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

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Received 30 December 2001; accepted in final form 10 June 2002

Reperfusion is the only means of salvaging ischemic myocardium and limiting infarct development. However, it is paradoxically associated with cell death termed lethal reperfusion injury (12, 19, 28). Proving the existence of lethal reperfusion injury is difficult, and the only way to investigate this phenomenon is by the administration of possible modulators of such injury immediately before reperfusion (but not before ischemia) with the subsequent assessment of infarct size. To date there is no experimental agent that has been shown to be effective in limiting lethal reperfusion injury both in animal models and the clinical setting. However, the clinical impact of such an intervention might be dramatic if it were used as an adjunct to thrombolysis at the time of reperfusion in the setting of acute myocardial infarction.

Recent studies suggest that p42/p44 MAPK and big MAPK (BMK1) (21, 22) are part of a “survival” pathway whereas p38 (specifically the p38α isoform) (17) and JNK mediate a “death” pathway in the ischemic-reperfused myocardium (2, 29). Yellon and Baxter (27) have suggested that p42/p44 MAPK pathway may represent an important salvage mechanism in the reperfused myocardium.

Urocortin (24) is a peptide related to the hypothalamic hormone corticotrophin-releasing factor (CRF) that was originally cloned from rat and thereafter the human brain and has receptors also expressed within peripheral tissues outside the central nervous system, including the heart (1, 10, 18). Urocortin has been shown to protect neonatal rat cardiomyocytes from cell death when administered before hypoxia or at the point of reoxygenation (3). Furthermore, urocortin has also been shown to induce p42/p44 MAPK phosphorylation in serum starved neonatal rat cardiomyocytes and inhibition of urocortin-mediated cardioprotection by the MEK1-p42/p44 MAPK-specific inhibitor PD-98059 suggests that this pathway may be important (3).

The aim of this study was to evaluate the infarct-limitation effects of urocortin when administered at reperfusion in adult rats both in isolated crystalloid-perfused hearts, and more importantly in an in vivo model of acute myocardial infarction. Furthermore, any possible infarct-altering hypotensive effects of urocortin in vivo (26) would be compared with a similar hypotensive effect caused by intermittent intravenous administration of nitrates at reperfusion. The role of p42/p44 MAPK in urocortin-induced cardioprotection was further evaluated both in the in vitro and in vivo setting to investigate the critical role for this MAPK in protecting against reperfusion injury. In addition, the specific effect of urocortin on p42/p44 MAPK phosphorylation and upregulation after ischemia-reperfusion was studied with the use of Western blot analysis.

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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-
Reperfusion injury and myocardial protection

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 ± 2.2% in control hearts. Determination of infarct size. At the end of each experiment, the hearts were rinsed with saline to remove the blood. The heart was excised and Langendorff perfused briefly with saline to remove the blood. The heart was excised and Langendorff perfused briefly with saline to remove the blood.

Results

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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.

Materials and methods. 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 ± 2.2% in control hearts.
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 cardio-protective 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.

Table 1. Rate pressure product analysis for all in vitro groups at time points throughout the ischemia-reperfusion protocol

<table>
<thead>
<tr>
<th>Groups</th>
<th>5 Min Stabilization</th>
<th>5 Min Ischemia</th>
<th>30 Min Ischemia</th>
<th>5 Min Reperfusion</th>
<th>60 Min Reperfusion</th>
<th>120 Min Reperfusion</th>
</tr>
</thead>
</table>

Values are means ± SE. The rate pressure product analysis for the in vitro groups is mmHg·beat·1·min.

4.3%. PD-98059 alone had no effect on infarct size compared with control (43.7 ± 1.8%).

RPP, CF rate, and ischemic risk zone analysis for in vitro experiments. The RPP (Table 1) and CF rate (Table 2) analysis showed there to be no significant difference between the groups at baseline (15-min stabilization). The RPP decreased significantly during ischemia in all groups, and there was only partial recovery during reperfusion. Urocortin had no adverse effect on cardiac function. The CF rate (expressed in ml·min⁻¹·100 g tissue⁻¹) was similar at baseline and decreased to a similar extent during regional ischemia. The ischemic risk volumes (zones) were similar within each group.

Effect of urocortin on p42/p44 MAPK phosphorylation. After the in vitro infarct size experiments, it was important to examine the effect of urocortin (and PD-98059) on p42/p44 MAPK phosphorylation throughout the ischemia-reperfusion period (Figs. 3 and 4). In the control experiments, p42/p44 MAPK phosphorylation was upregulated at all time points measured compared with the stabilized preparation, with greatest activation seen at 5-min reperfusion (Fig. 3). After the administration of urocortin at reperfusion (Fig. 3), there was a significant upregulation of the p42/p44 MAPK signaling pathway, with maximal induction of phospho-p42/p44 MAPK occurring at 10-min reperfusion compared with control values. Interestingly, at 20-min reperfusion, the level of phospho-p42/p44 MAPK in the urocortin-treated group was lower than that at 10-min reperfusion despite the presence of urocortin, although still significantly greater than its time-matched control. By 2-h reperfusion, there was no difference between the urocortin-treated and control groups. In the groups treated with urocortin + PD-98059 or PD-98059 alone (Fig. 4), no significant difference was noted in phospho-p42/p44 MAPK levels compared with their time-matched controls (at 10-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 ± 0.5</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>326 ± 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 PO\textsubscript{2} 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 \(\mu\)g/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 upregulation may protect the myocardium against the consequences of reperfusion injury. p42/p44 MAPK and BMK1 have been shown to protect against ischemia-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-
phorylation (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 crystalloid 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 CF 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 differences in the endocrine system, as urocortin treatment in other species has been shown to be associated with increased ACTH levels (26). Thus, urocortin may play a role in the hemodynamic effects of urocortin in vivo by modulating the peripheral vasoconstrictor tone and reducing the cardiac output during the reperfusion phase.

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

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