Urocortin Protects against Ischemic and Reperfusion Injury via a MAPK-dependent Pathway*

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Urocortin (UCN) is a peptide related to hypothalamic corticotropin-releasing hormone and binds with high affinity to corticotropin-releasing hormone receptor-2β, which is expressed in the heart. In this study, we report that UCN prevented cell death when administered to primary cardiac myocyte cultures both prior to simulated hypoxia/ischemia and at the point of reoxygenation after simulated hypoxia/ischemia. UCN-mediated cell survival was measured by trypan blue exclusion, 5′-OH end labeling of DNA (TUNEL), annexin V, and fluorescence-activated cell sorting. To explore the mechanisms that could be responsible for this effect, we investigated the involvement of MAPK-dependent pathways. UCN caused rapid phosphorylation of ERK1/2-p42/44, and PD98059, which blocks the MEK1-ERK1/2-p42/44 signaling cascade, also inhibited the survival-promoting effect of UCN. Most important, UCN reduced damage in isolated rat hearts ex vivo subjected to regional ischemia/reperfusion, with the protective effect being observed when UCN was given either prior to ischemia or at the time of reperfusion after ischemia. This suggests a novel function of UCN as a cardioprotective agent that could act when given after ischemia, at reperfusion.

Urocortin (UCN) is a peptide related to hypothalamic hormone corticotropin-releasing factor (CRF), the central mediator of the hypothalamic-pituitary-adrenal axis and stress response in mammals (1–3). UCN and CRF share 45% homology at the amino acid level, and both are synthesized as precursors, which are subsequently processed to the mature biologically active peptides (in the case of UCN, a 40-amino acid molecule) (4, 5). Although UCN was originally identified in restricted areas of the brain, it has also been found in the placenta, lymphocytes, and heart (6–9).

The CRF family of peptides bind two types of CRF receptors, CRF-R1 and CRF-R2. CRF-R2 exists in three alternative splice variant forms, CRF-R2α, CRF-R2β, and CRF-R2γ (10–12); and CRF-R2 binds UCN with a higher affinity than CRF both in ligand binding studies (4) and as shown by the effects of ligand on intracellular cAMP (13). In contrast, the R1 receptors show little ligand selectivity for UCN versus CRF. R2 receptors are the only type of CRF receptor found in the heart: the α form in man (14) and the β form in the rat (15). The CRF family of peptides have been shown to stimulate adenylate cyclase activity in cardiac myocytes (16), and changes in CRF-R2 expression have been reported in the hearts of spontaneously hypertensive rats (17).

The coincident expression of CRF-R2 receptors with their preferred UCN ligand in the heart suggests that UCN may have physiological cardiac properties. Indeed, UCN, but not CRF, induces a dose-dependent increase in heart rate, cardiac output, and coronary blood flow (18). Moreover, cardiac CRF-R2β expression is modulated by endotoxin, a potent inducer of cardiovascular dysregulation, further suggesting a possible link between UCN and the cardiovascular response to stress (19). Indeed, in previous studies, we have shown that UCN mRNA expression increases in cardiac cells exposed to thermal and simulated hypoxic/ischemic injury stress in vitro (9) and that endogenous UCN peptide protects cardiac myocytes from cell death when administered prior to the stress (20).

A number of studies have implicated the mitogen-activated protein kinase (MAPK) pathway as a survival pathway in both cardiac cells (21–23) and other cell types (24–29). The extracellular signal-related kinases (ERKs), belonging to one subfamily of MAPKs, are composed of 42- and 44-kDa kinases named p42 ERK and p44 ERK, respectively. Phosphorylation and activation of p42 ERK and p44 ERK are mediated by the MAPKs MEK1 and MEK2.

CRF has been shown to activate ERK1/2-p42/44 in Chinese hamster ovary cell lines transfected with CRF-R1 and CRF-R2 (30). Moreover, the cardioprotective effects of cardiotophin-1 (CT-1) are mediated through gp130 receptor activation of the MEK1/2-ERK1/2-p42/44 signaling cascade, one consequence of which is increased expression of the cardioprotective hsp70 and hsp90 proteins (31).

In this report, we have extended our studies on the cardioprotective effects of UCN in primary cultures of neonatal rat cardiac myocytes exposed to simulated ischemia/reoxygenation in vitro and show that UCN is also protective in isolated perfused rat hearts ex vivo. In both in vitro and ex vivo models, we show for the first time that this cardioprotective effect of UCN is retained when addition of UCN is delayed until after the simulated hypoxia/ischemia and is given during the reoxygenation/
reperfusion. We also show that the protective effects involve activation of the MEK1/2-ERK1/2-p42/44 signaling pathway.

**MATERIALS AND METHODS**

**Antibodies and Reagents**—Anti-active MAPK polyclonal antibody and rabbit anti-ERK1/2-p42/44 antibody were obtained from Promega, as were anti-active p38 MAPK polyclonal antibody and rabbit anti-active-JNK polyclonal antibody. Anti-actin goat polyclonal antibody was supplied by Santa Cruz Biotechnology. Mouse monoclonal antibody to dennin was obtained from Sigma (Dorset, United Kingdom). Secondary antibodies used were horseradish peroxidase-conjugated anti-mouse and anti-actin IgG and anti-actin IgG, which were obtained from Dako A/s (Denmark House, Cambridge, U.K.). For Western blot analysis, all primary antibodies were used at a dilution of 1:1000. Secondary antibodies were used at a dilution of 1:2000.

Recombinant rat UCN peptide was obtained from Sigma. In all experiments, UCN was used at a concentration of 10^{-7} m. The MEK1/2 inhibitor PD98059 was purchased from New England Biolabs Inc. (Beverly, MA) and has been shown to act as a highly selective inhibitor of MEK1 activation and the MAPK ERK1/2-p42/44 cascade (32).

Reagents for assessing apoptotic nuclei by label deling of DNA 3'-OH ends with fluorescein isothiocyanate (FITC)-conjugated 2'-deoxyuridine-5'-triphosphate and terminal transferase were purchased from Roche Molecular Biochemicals. FITC-annexin V conjugate and -triphosphate and terminal transferase were purchased in a humidified atmosphere of 5% CO2, 0% O2, and balance gas N2 at a pressure of 4 pounds/inch^2. Untreated cells were cultured in Esumi control buffer in a humidified atmosphere of 5% CO2, 21% O2 at 37 °C for 6 h as internal controls for this experiment. Modified Esumi control buffer contained 137 mM NaCl, 0.9 mM CaCl2, 0.4 mM HEPES, 10 mM dextrose, and 20 mM sodium lactate (pH 6.2) (34).

Normal growth medium was replaced with serum-free medium for 24 h. UCN was added to the cardiac myocytes immediately and after 1, 6, and 24 h prior to the lethal simulated hypoxia/ischemic insult. Subsequent to this injury, cell survival was assessed by trypan blue exclusion. The MEK1 inhibitor PD98059 was incubated with the cardiac myocytes in serum-free medium 10 min prior to a 30-min or 24-h incubation of UCN in a normoxic environment. Subsequently, the cells were exposed to a 6-h lethal simulated hypoxic/ischemic insult, and cell injury was assessed by the end labeling of DNA 3'-OH ends with FITC-dUTP, trypan blue exclusion, and FACS analysis for the 24-h UCN incubation period. For annexin V labeling, cells were exposed to only a 2-h ischemic insult as annexin V binds to phosphatidylserine residues that move from the inner membrane to the outer cell membrane during the initial stage of apoptosis.

**Trypan Blue Exclusion**—The cells were harvested as described previously (39). Following the addition of an equal volume of 0.4% trypan blue in PBS, the percentage of blue cells/total cells was counted by scoring 250 cells, three times per well, using a hemocytometer.

**TUNEL**—Apoptotic nuclei were assessed by the end labeling of DNA 3'-OH ends with FITC-dUTP using a modification of the TUNEL method as described previously (38, 39). TUNEL-positive cells were scored by fluorescent microscopy. The percentage of apoptotic nuclei is expressed as a percentage of total nuclei from scoring 250 cells, three times per well.

**FACS Analysis**—Cells were incubated with and without UCN in the presence and absence of PD98059 for 24 h prior to exposure to lethal simulated hypoxia/ischemia. Apoptosis and cell death were evaluated by FACS as described previously (n = 3 for each treatment) (31, 40). Briefly, 10^5 cells were pelleted by centrifugation at 1200 rpm for 5 min and resuspended in 500 μl of methanol/aceton (4:1) overnight at 4 °C. The cells were then pelleted and resuspended in 50 μl of RNase (20 μg/ml) for 15 min at 37 °C. Subsequent to this incubation period, 100 μl of propidium iodide (40 μg/ml) was added to the cells, which were then incubated in the dark for 20 min at 37 °C. DNA fluorescence was measured using a FACScan (Becton-Dickinson). Nuclei were gated according to fluorescence. The M1 gate detects cells undergoing apoptosis/cell death. The M2 gate detects live cells, i.e., cells in G1.

**Annexin V Labeling**—Cells were incubated, as before, in serum-free medium for 24 h and cultured for 10 min with PD98059 prior to a 30-min or 24-h incubation with UCN. The cells were then exposed to a 2-h ischemic insult and labeled as described previously (39, 41). Briefly, the ischemic buffer was removed, and the cells were washed with PBS. To each of the wells was added 10 μl of FITC-conjugated human annexin V (10 μg/ml) in 90 μl of binding buffer concentrate (HEPES-buffered saline solution supplemented with 25 mM CaCl2). The cells were incubated at room temperature in the dark for 1 h on a swirling base. The annexin V label was washed, and the cells were carefully removed by centrifugation, resuspended in PBS, and imaged by fluorescent microscopy. Following mixing with 3% (v/v) paraformaldehyde for 30 min at 25 °C, the cells were washed three times with PBS. Annexin V-positive cells are expressed as a percentage of total cells (observed under phase). A minimum of 250 cells were scored, three times per well.

**Measurement of Cardioprotective Effects of UCN in Reoxygenation after Simulated Hypoxia Ischemia**—To investigate the downstream signaling pathways that mediate UCN cardioprotection in reoxygenation...
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Cardiovascular injury, the ischemic buffer was removed from the cardiac myocytes at the point of reoxygenation and replaced with 1 ml of serum-free medium with and without the addition of UCN and PD98059 for 2 h. PD98059 was added to the cardiac cells 10 min prior to the addition of UCN. Following a 2-h reoxygenation period, the cardiac myocytes were assessed for cell survival by trypan blue exclusion, TUNEL, and annexin V labeling as described above.

Isolated Rat Heart Preparation (Langendorff Perfusion)—Twenty-three male Wistar rats (250-350 g) fed a standard diet were heparinized (200 IU) and anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg). The hearts were excised, placed in ice-cold Krebs-Henseleit buffer, and rapidly mounted on the aortic cannula of a Langendorff perfusion system. Perfusion was established within 30 s after thoracotomy. The Krebs-Henseleit buffer (pH 7.4, 95% O₂ and 5% CO₂) contained 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄.₈ 1.8 mM CaCl₂·2H₂O, 25.2 mM NaHCO₃, and 11 mM glucose. Perfusion pressure was maintained at 100 cm H₂O and the myocardial temperature was kept constant at 37 °C. A water-filled latex balloon, connected to a hydrostatic pressure transducer (P23XL, Viggo-Spectramed) and coupled to a recorder (Multitrace 2, Lexomed), was inserted into the left ventricle through an incision in the left atrium and inflated to set an end diastolic pressure of 5–10 mm Hg. Coronary flow was measured by timed collection of effluent over 1 min at each sampling point. A 3-0 silk suture was passed around the main branch of the left coronary artery, and the ends were threaded through a small vinyl tube to form a snare. Regional ischemia was achieved by pulling the snare and confirmed by a substantial fall in both left ventricular developed pressure and coronary flow. All hearts underwent 20 min of stabilization before being subjected to 35 min of regional ischemia and 2 h of reperfusion. Base-line values for functional parameters were obtained after 10 min of perfusion. Three groups were included in the study. The first group served as a control (n = 8); the second group was treated with 10⁻⁷ M UCN (n = 7) 30 min prior to ischemia; and the third group received UCN for 30 min from the onset of reperfusion (n = 8). At the end of each experiment, the silk suture was reooled, and a 0.5% suspension of zinc-cadmium sulfide fluorescent particles (1–10 μm in diameter; Duke Scientific Corp., Palo Alto, CA) was infused into the perfusate to mark the risk zone as non-fluorescent tissue. The hearts were then frozen and cut into 2-mm thick slices parallel to the atrio-ventricular groove. The slices were thawed and incubated with 1% triphenyltetrazolium chloride in phosphate buffer (pH 7.4) at 37 °C for 20 min and fixed in 10% Formalin to enhance the contrast of the stain. Slices were then compressed to a uniform 2-mm thickness by placing them between two glass plates separated by a 2.0-mm spacer. The area of the left ventricle, the infarcted area (triphenyltetrazolium chloride stain-negative), and the risk zone (non-fluorescent under UV light) were traced onto acetate transparency using a computerized planimetry program (Summa Sketch II, Summa Graphics, Seymour, CT). The infarct size and the risk zone areas were calculated for each heart slice and the product. Infarct size was expressed as a percentage of the risk zone. These measurements were performed in a blinded fashion. Heart rate, coronary blood flow, and left ventricular developed pressure were measured at 10-min intervals throughout the experiments.

Statistics—Data for in vitro experiments are expressed as means ± S.D. Single-factor one-way analysis of variance (ANOVA) was performed for each group of treatments, and significance was assumed when the p value was <0.05. Differences among means were compared within the treatment groups using Student’s t test. The experiments were repeated at least three times for each experiment as stated in the legends; each n value corresponds to the mean of three random fields/well of cells with a minimum of 250 cells scored per view. The n value for each experiment is stated under “Results.” Infarct size data were tested for group differences by ANOVA combined with Tukey’s post hoc test. Comparisons of cardiac flow, heart rate, and left ventricular developed pressure were performed with repeated measures of ANOVA. p values <0.05, <0.01, <0.005 were considered significant.

RESULTS

Cardioprotective Effects of UCN Administered 0 and 30 min and 1, 2, and 24 h before Lethal Simulated Hypoxic/Ischemic Injury—UCN was added to the normal growth medium of cardiac myocytes 0 and 30 min and 1, 2, and 24 h prior to lethal simulated hypoxic/ischemic injury, and cell death was assessed by trypan blue exclusion. The percentage of dead cells in cultures incubated in a normoxic environment was 16.9% (n = 6). Cardioprotection was not detected when UCN was added immediately and removed immediately from the cells, as the percentage of cell death was 60.8% (n = 8) compared with 65.5% (n = 8) of cell death measured in ischemic untreated cells. However, the percentage of cell survival increased when UCN was incubated for the 30-min time period and for longer periods. Cell death decreased from 65.5 to 42.5% (n = 6), 44.4% (n = 6), 42.1% (n = 10), and 47.6% (n = 6) for pretreatment times of 30 min (*, p < 0.03), 1 h (**, p < 0.002), 2 h (***, p < 0.001), and 24 h (****, p < 0.001), respectively. No significant increase in cell survival was measured comparing the 30-min and 24-h pretreatments with UCN (Fig. 1). Hence, a 30-min pretreatment with UCN is sufficient for its protective effect.

Phosphorylation of ERK1/2 by UCN—The rapid protective effect of UCN led us to investigate the signaling pathways that it activates and that may reduce its protective effect. CRF has been shown to activate the MEK1-ERK1/2-p42/44 signaling cascade in Chinese hamster ovary cells overexpressing CRF-R1 and CRF-R2 (30). Activation of this signaling pathway mediates the anti-apoptotic effect of CT-1, indicating that it can be protective (21). To study the effects of UCN on ERK1/2 phosphorylation, cardiac myocytes were serum-starved for 24 h and treated with UCN for 10 min and 1, 6, and 24 h. Untreated and UCN-treated cells were harvested, and total cellular proteins were subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis (see “Materials and Methods”) using a antibody specific for phosphorylated ERK1 and ERK2 (p42/44), which migrate at molecular masses of 42 and 44 kDa, respectively. ERK1 and ERK2 (p42/44) were rapidly phosphorylated within 10 min and remained phosphorylated at 1 and 6 h; however, at 24 h, phosphorylated enzyme returned to basal levels (Fig. 2). The phosphorylated levels of ERK1/p42 appear to be greater than those of ERK2/p44 following UCN treatment. No increased phosphorylation of JNK and p38 MAPK was detected after UCN treatment (data not shown). The levels of actin protein were also constant. To our knowledge, this is

![Figure 1](http://www.jbc.org/)

**FIG. 1.** Cardioprotective effects of UCN administered before lethal simulated hypoxic/ischemic injury. UCN was added to the normal growth medium of cardiac myocytes 0 and 30 min and 1, 2, and 24 h prior to lethal simulated hypoxic/ischemic injury, and cell death was assessed by trypan blue exclusion. The percentage of dead cells in cultures incubated in a normoxic environment was 16.9% (n = 6). Single-factor ANOVA was performed on the group of treatments that showed highly significant differences (p value = 2.42 × 10⁻⁶). Cell death decreased from 65.5 to 42.5, 44.4, 42.1, and 47.6% for pretreatment times of 30 min (*, p < 0.03), 1 h (**, p < 0.002), 2 h (***, p < 0.001), and 24 h (****, p < 0.001), respectively. NT, no treatment.
the first report that UCN activates the MAPK ERK1/2-p42/44 signaling cascade, as has previously been demonstrated for CRF.

**UCN Protection Is Abolished by the MEK1 Inhibitor PD98059—**MEK1 and MEK2 are upstream activators of the p42/44 MAPKs whose activity is inhibited by PD98059 (32). Cardiac myocytes were pretreated for 10 min with PD98059 and cultured with UCN for 30 min and 24 h prior to exposure to lethal simulated hypoxia/ischemia. Cell death was assessed by trypan blue exclusion (Table I). A 30-min pretreatment with UCN prior to ischemia reduced cell death from 75.65% (n = 6) to 45.66% (n = 6; p < 0.016); and UCN cardioprotection was inhibited by pretreatment with PD98059, as cell death increased to 69.25% (n = 6; p < 0.013). The 24-h pretreatment with UCN prior to ischemia reduced cell death from 62.22% (n = 12) to 47.43% (n = 11; p < 0.0001); and UCN cardioprotection was inhibited by pretreatment with PD98059, as cell death increased to 62.55% (n = 11; p < 0.00004). PD98059 administered on its own had no effect on hypoxia/ischemia-induced cell death in both 30-min and 24-h pretreatment experiments, as the percentages of cell death were 64.4 and 62.45%, respectively. Hence, UCN protection against simulated hypoxia/ischemia-induced death is inhibited by the MEK1 inhibitor.

**UCN-mediated Cardioprotection Measured by Annexin V, TUNEL, and FACS—**Cell injury was assessed using marker techniques for apoptosis such as annexin V, TUNEL, and FACS analysis. The percentages of dead cells measured by TUNEL, annexin V, and FACS in a normoxic environment prior to the experiment were 10.48% (n = 7), 5.7% (n = 5), and 24.98% (n = 3), respectively. The variation in cell death under normoxic conditions using the different techniques may be accounted for by their different sensitivity and detection of specific markers in the cell death pathway. Since both TUNEL and FACS assess DNA cleavage, it is possible that preparation of cells for FACS may cause some cell damage. However, as seen for trypan blue exclusion, the 30-min pretreatment with UCN produced a cardioprotective effect as observed using annexin V labeling and TUNEL (Table I). Cell injury, as measured by annexin V labeling, was reduced from 44.99% (n = 6) in untreated cells to 29.54% (n = 6; p = 0.04) in UCN-treated cells. UCN-mediated cardioprotection was inhibited by pretreatment with PD98059 prior to UCN, as cell injury increased to 37.60% (n = 6). Cell injury, as measured by TUNEL, was reduced from 37.2% (n = 6) in untreated cells to 30.66% (n = 6) in UCN-treated cells. UCN-mediated cardioprotection was inhibited by pretreatment with PD98059 prior to UCN, as cell injury increased to 39.0% (n = 6).

**FACS analysis was performed on untreated cardiac cells and those treated with UCN for 24 h with and without PD98059.** Lethal simulated hypoxia/ischemia resulted in 94.76% cell injury/death (M1 gate), which accounts for cells undergoing apoptosis. Incubation of PD98059 alone had no effect on cell survival when administered during the stress. The addition of UCN resulted in a decrease in cell death to 56.34% (p < 0.001), and PD98059 partially abrogated the UCN-mediated cell survival effect to increase cell death to 75.56% (p < 0.041). Hence, UCN activates MEK1, which results in the serine/threonine phosphorylation of factors that mediate the survival effect of UCN.

**UCN Protects Cardiac Myocytes from Reoxygenation Injury, and This Effect Is Partially Inhibited by PD98059—**To assess whether UCN retains cardioprotective effects when added after a period of simulated hypoxia/ischemia, UCN was added to the cardiac myocytes at the point of reoxygenation (Table II). Cell death was measured in untreated cells exposed to ischemic reoxygenation by trypan blue exclusion, TUNEL, and annexin V. UCN protected the cardiac cells during reoxygenation when cell death was assessed by trypan blue exclusion (n = 8; p < 0.00025), TUNEL (n = 8; p < 0.0017), and annexin V (n = 6; p < 0.004) compared with untreated cells exposed to ischemic reoxygenation. To investigate whether UCN protection at reoxygenation was mediated by the MEK1-ERK1/2-p42/44 signaling cascade, PD98059 was administered for 10 min prior to the addition of UCN. The cardioprotective effect of UCN was inhibited by PD98059 when cell death was assessed by trypan blue exclusion (n = 8; p < 0.00008) and annexin V (n = 7; p < 0.00015). PD98059 alone had no effect on cell injury during reoxygenation. An inhibitory effect of PD98059 on UCN-mediated protection in simulated ischemic/hypoxic injury was also observed using TUNEL.

**UCN Reduces Infarct Size of the Intact Rat Heart when Administered before and after a Simulated Ischemic Insult—**Having shown protective effects of UCN on simulated hypoxic/ischemic stress Cardiac myocytes were pretreated with PD98059 (10 min) and cultured with UCN for 30 min and 24 h prior to exposure to lethal simulated ischemia (I). Cell death was assessed by trypan blue exclusion, annexin V labeling, and TUNEL. A 30-min pretreatment with UCN prior to lethal simulated hypoxia/ischemia was sufficient to reduce cell death as measured by trypan blue exclusion and annexin V labeling. UCN-mediated cardioprotection was inhibited by pretreatment with PD98059 prior to UCN when cell death was measured by trypan blue exclusion. PD98059 abrogated the cardioprotective effects of UCN when cell survival was assessed by annexin V labeling and TUNEL. NT, no treatment.

**Table I**  
Assessment of percent cell death, annexin V-positive cells, and TUNEL-positive nuclei following pretreatment of cardiac myocytes with UCN prior to simulated hypoxic/ischemic stress

| Treatment time with UCN | Method of assessing cell viability | NT, I (%) | UCN, I (%) | UCN + PD98059, I (%) | PD98059, I (%) | p value (ANOVA) |
|-------------------------|----------------------------------|-----------|-----------|----------------------|---------------|----------------|
| 30 min                  | Trypan blue                      | 75.65 ± 5.87 | 45.66 ± 8.00 | 69.26 ± 6.10         | 64.41 ± 9.99  | 0.0008         |
|                         |                                   |           | (p < 0.016) | (p < 0.013)          |               |                |
| 24 h                    | Trypan blue                      | 62.23 ± 7.60 | 47.43 ± 6.14 | 62.56 ± 4.88         | 62.40 ± 8.98  | 6.8 × 10⁻⁶      |
|                         |                                   |           | (p < 0.000) | (p < 0.000)          |               |                |
| 30 min                  | Annexin V                        | 44.99 ± 9.10 | 29.54 ± 8.49 | 37.60 ± 6.98         | 42.99 ± 5.28  | 0.01           |
|                         |                                   |           | (p < 0.05)  | (p < 0.05)           |               |                |
| 30 min                  | TUNEL                            | 37.2 ± 10.19 | 30.07 ± 7.59 | 39.00 ± 10.18        | 33.47 ± 7.82  | 0.35           |
|                         |                                   |           |             |                      |               |                |

Fig. 2. Phosphorylation of ERK1/2-p42/44 by UCN. Cardiac myocytes were serum-starved for 24 h and treated with UCN for 10 min (n) and 1, 6, and 24 h. Cells with no treatment (NT) and UCN-treated cells were harvested, and total cellular proteins were subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis using an antibody specific for phosphorylated ERK1 and ERK2 (p42/44), which migrate at molecular masses of 42 and 44 kDa. ERK1 and ERK2 were rapidly phosphorylated within 10 min and remained phosphorylated for 6 h; however, at 24 h, phosphorylated enzyme returned to basal levels.
Cardiac myocytes were exposed to lethal ischemic/hypoxic insult, and UCN was added to the cardiac myocytes at the point of reoxygenation (IR). UCN protected the cardiac cells during reoxygenation when cell death was assessed by trypan blue exclusion (p < 0.00008), TUNEL (p < 0.0017), and annexin V (p < 0.0004) compared with the untreated cells exposed to reoxygenation. The cardioprotective effect of UCN was inhibited by PD98059 when cell death was assessed by trypan blue exclusion (p < 0.00008) and annexin V (p < 0.00015). PD98059 alone had no effect on cell injury during reoxygenation. NT, no treatment.

| Method of assessing cell viability | NT, IR | UCN, IR | UCN + PD98059, IR | PD98059, IR | p value (ANOVA) |
|----------------------------------|--------|---------|-----------------|-------------|----------------|
| Trypan blue                      | 56.47 ± 8.85 | 32.62 ± 5.93 | 48.68 ± 3.28 | 52.03 ± 3.32 | 4.32 × 10⁻⁷ |
| (p = 0.0003)                     |         |         | (p = 0.00008)  |             |                |
| Annexin V                        | 50.98 ± 9.29 | 30.36 ± 2.79 | 41.34 ± 4.56 | 42.16 ± 7.68 | 8.68 × 10⁻⁵ |
| (p = 0.004)                      |         |         | (p = 0.0015)  |             |                |
| TUNEL                            | 43.26 ± 5.41 | 29.75 ± 4.67 | 56.22 ± 12.10 | 35.52 ± 6.70 | 3.42 × 10⁻⁵ |
| (p = 0.0017)                     |         |         |                 |             |                |

DISCUSSION

Cardiac muscle survival is of critical importance in maintaining the normal function of the heart. Adult cardiac muscle cells are terminally differentiated and have lost their ability to proliferate. Therefore, irreversible cardiac injury, e.g. following ischemia, results in scarring and eventual decrease in cardiac function. Identification of natural cardioprotective agents and the signaling pathways through which their cardioprotective effects are mediated is critical for the elucidation of the molecular basis of cardiac myocyte loss and rescue.

In this study, we have extended our previous observations on the cardioprotective properties of UCN. In in vitro cultures of primary rat cardiac myocytes exposed to simulated hypoxia/ischemia followed by reoxygenation, exogenous UCN reduces cardiac myocyte cell death when added after the simulated hypoxia/ischemia and during the reoxygenation/reperfusion period as well as when added prior to the simulated hypoxia/ischemia (20). Moreover, in the ischemic/reperfused isolated heart ex vivo, UCN is also cardioprotective when added to the perfusate after ischemia and during reperfusion. Since clinical intervention is possible only after the acute ischemic episode, the data suggest that UCN and derivatives thereof may have a role in the management of human myocardial infarction.

We have previously shown that UCN mRNA is expressed by both cultured neonatal rat cardiac myocytes and the adult rat heart, and not CRF, and that UCN transcripts are increased following simulated hypoxia/ischemia (20). By Western blotting, we have also demonstrated the presence of the 22-kDa UCN precursor protein in cardiac myocytes (data not shown) and that the mature 40-amino acid UCN peptide is released into the supernatant of cardiac myocytes exposed to simulated hypoxia/ischemia. This is consistent with the cardioprotective effects of these supernatants being inhibited by CRFα9–41, a receptor antagonist of all CRF family members at both CRF-R1 and CRF-R2, and suggests that endogenous cardiac UCN is released from ischemic cardiac myocytes and exerts an autocrine/paracrine protective effect through cardiac CRF-R2. We have also shown that UCN protects cardiac myocytes from ceramide-induced apoptosis.2 There is increasing evidence that UCN and CRF peptides are involved in cell survival mechanisms of a number of cells. CRF administered to hypoxic rat brain slices results in neuroprotection when administered during the hypoxic episode (42). The mechanism of action appears to be a direct neuronal effect. Therefore, we suggest that UCN peptide release may serve as a mechanism to protect cells from hypoxia/ischemia-induced cell death.

A number of studies have addressed which signaling and transactivating pathways are activated by ischemia and ische-

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2 B. K. Brar, A. K. Jonassen, A. Stephanou, G. Santilli, J. Railson, R. A. Knight, D. M. Yellon, and D. S. Latchman, unpublished observations.
mia/reperfusion. In the heart, the p38 MAPKs and JNKs are induced by ischemia and ischemia/reperfusion (43–45, 47–49). The JNK and p38 stress-activated pathways have been linked to an increase in cell death in ischemic and reoxygenated cardiac cells (49). UCN failed to stimulate the phosphorylation and activation of both p38 and JNK. A highly specific inhibitor of p38 MAPK (SB203580) failed to inhibit the cardioprotective effect of UCN in all experiments (data not shown). Our experiments show that UCN induces ERK1/2-p42/p44 phosphorylation and that inhibition of MEK1/2, the upstream activator of these enzymes, inhibits the cardioprotective effect of UCN. The target cardioprotective genes or the post-translational events induced by UCN-mediated activation of ERK1/2-p42/p44 are unknown. However, in other cells, ERK1 and ERK2 (p42/p44) have been associated with the increased expression of FLICE (FADD-like interleukin 1b-converting enzyme) like inhibitory protein, an inhibitor of the caspase cascade (50). Whether UCN mediates its cardioprotection by increasing the levels of FLICE-like inhibitory protein has yet to be determined.

It is known that both hypoxia/ischemia and reoxygenation impose redox stress on cardiac tissue. Although coronary reperfusion itself results in tissue injury (51), it is the only means of limiting infant heart size, provided that it occurs early after coronary occlusion. In this study, we report for the first time that UCN reduces damage to an intact rat heart exposed to regional ischemia when given at reperfusion, which would be essential in the clinical setting. Growth factors such as insulin have been shown to exert anti-apoptotic effects in these cells when administered during reperfusion. Insulin-like growth factor I (52) and insulin (53) have been shown to inhibit apoptosis and necrosis in cardiac myocytes. Both insulin-like growth factor I and insulin act through phosphatidylinositol 3-kinase and receptor tyrosine kinases as well as the MEK1-ERK1/2-p42/p44 signaling cascade (54). The benefit of UCN over these and other drugs will need to be investigated in the intact animal and human tissue.

Some cardioprotective factors such as CT-1 (55) and angiotensin II and vasopressin (56) are also potent inducers of hypotension; hence, it acts as a hypotensive agent (59). Whether UCN caused a significant decrease in mean arterial blood pressure in cardiac myocytes induced by cellular stressors warrants further investigation.

UCN peptide will enable accurate measurement of UCN release and expression in our models of hypoxic/ischemic and hypoxic/ischemic/reperfusion injury and in future human studies (46). It is already clear, however, that as well as the adaptive role in the response to external stressors, UCN is also an important mediator of the response to stress at the level of the cell. We believe that these results establish a new and potentially clinically important role for UCN in protection from cellular stress, in addition to the well known function of the CRF family of peptides in integrating the complex response to external physical and psychological stresses. The use of these peptides in salvaging neurons and other cell types that express CRF receptors, in addition to cardiac myocytes, from cell death induced by cellular stressors warrants further investigation.
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