Urocortin prevents mitochondrial permeability transition in response to reperfusion injury indirectly by reducing oxidative stress

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UCN treatment protects the heart by inhibiting MPTP opening.

The level of oxidative stress during reperfusion was reduced in mitochondria also exhibited less protein carbonylation, suggesting that UCN decreases levels of oxidative stress. In isolated adult and neonatal rat cardiac myocytes, both acute (60 min) and chronic (16 h) treatment with UCN reduced cell death following simulated ischemia and re-oxygenation. This was accompanied by less MPTP opening as measured using tetramethylrhodamine methyl ester. The oxidative stress during reperfusion was reduced in cells that had been pretreated with UCN, suggesting that this is the mechanism by which UCN desensitizes the MPTP to reperfusion injury. Despite the fact that we could find no evidence that either PKC-ε or PKC-α translocate to the mitochondria following acute UCN treatment, inhibition of PKC with chelerythrine eliminated the effect of UCN on oxidative stress. Our data suggest that acute UCN treatment protects the heart by inhibiting MPTP opening. However, the mechanism appears to be indirect, involving a PKC-mediated reduction in oxidative stress.

THE IDENTIFICATION AND CHARACTERIZATION of agents that can protect the heart from the damaging effects of ischemia-reperfusion (I/R) are of considerable importance. Such agents could be given as part of cardiac surgery procedures either to minimize the effects of ischemic damage during cardiac surgery or, in the case of transplantation, during transportation of the heart. Similarly, if such agents are protective when given at reperfusion following ischemia, they could be used therapeutically to minimize the effects of reperfusion injury following an ischemic episode treated with angioplasty or thrombolytic agents.

Urocortin (UCN) is a forty amino acid peptide that is closely related to corticotrophin-releasing factor (CRF) (for review, see Ref. 27). We have demonstrated previously that the addition of exogenous UCN reduces the amount of cell death in cultured cardiac cells exposed to hypoxia/reoxygenation (6, 7, 36). Moreover, this protective effect of UCN can also be observed in the intact heart: an addition of UCN to a Langendorff-perfused heart preparation reduces the infarct size induced by I/R and strongly enhances posts ischemic recovery of cardiac function (6, 39). UCN has been shown to bind to two distinct G protein-coupled receptors, CRF-R1 and CRF-R2. However, the CRF-R1 receptor is not expressed in the heart, making it likely that the cardioprotective effects of UCN are mediated by its binding to CRF-R2 (26, 42).

Both in culture and in the intact heart, the protective effects of UCN on cardiac function can be demonstrated when UCN is given at reperfusion following the ischemic episode (6, 39). This indicates that UCN can minimize reperfusion injury, as well as damage occurring during ischemia, and may therefore be beneficial therapeutically if given at reperfusion following an ischemic episode. Such a protective effect of UCN at reperfusion was also observed in an initial in vivo study in which we showed that UCN could reduce infarct size when injected into the intact heart 3 min before the end of a 25-min ischemic period (40). Moreover, although UCN also produced a fall in blood pressure in this study, in accordance with previous reports (37), this was not the reason for its cardioprotective effect since no cardioprotection resulted from an equivalent reduction in blood pressure obtained using a hypotensive agent (40).

In view of the clear protective effect of UCN and its potential therapeutic importance, we have recently investigated the mechanisms underlying the protection it affords. Using Affymetrix gene chip technology and subsequent Western blot analysis, we have demonstrated that UCN can induce the expression of several proteins that have been implicated in cardioprotection, such as the Kir6.1 potassium channel subunit and PKC-ε while repressing the expression of calcium-independent phospholipase A2 (28–30). Blocking each of these changes pharmacologically inhibited the cardioprotective
effect of UCN in both cultured cardiac cells and Langendorff-perfused hearts. Recently, we have used the measurement of mitochondrial membrane potential in cultured cardiac myocytes to demonstrate that UCN can prevent the damaging effect of I/R on mitochondria in vitro (31). Taken together, these data implicate mitochondria as a major target for the protective effects of UCN, as is also the case for a range of other protective regimes, including pre- and postconditioning (15, 18, 44).

In recent years it has become increasingly apparent that a critical process in reperfusion injury is the opening of the mitochondrial permeability transition pore (MPTP) (15). This nonspecific channel in the inner mitochondrial channel opens under conditions of elevated mitochondrial calcium, especially when associated with oxidative stress and adenine nucleotide depletion. These are exactly the conditions that pertain during reperfusion following a period of ischemia. Indeed, the opening of the pore during reperfusion has been demonstrated experimentally, while inhibitors of the MPTP, such as cyclosporin A (CsA) and sanglifehrin A, can protect the heart from reperfusion injury (15, 44). There is increasing evidence that protective regimes such as ischemic pre- and postconditioning, as well as mimics such as adenosine, PKC agonists, and ATP-sensitive potassium channel openers, operate through inhibition of MPTP opening, although the signaling pathways involved are unclear (11, 15, 18, 19). Some workers have argued that inhibition is mediated by a direct phosphorylation of components of the MPTP, perhaps involving translocation of PKC-ε or glycogen synthase kinase 3 to the mitochondria (2, 22). By contrast, others, including ourselves, have provided data to suggest that protection is secondary to a reduction in oxidative stress and calcium overload (20).

Here we use the Langendorff-perfused heart models of I/R to provide the first evidence showing that UCN can inhibit MPTP opening in the intact heart and investigate the mechanisms involved in preventing MPTP opening upon reperfusion in both the cultured cardiac myocyte and the Langendorff models. Our data imply that UCN protects hearts from reperfusion injury by inhibiting MPTP opening through an indirect mechanism, such as reduced oxidative stress and/or calcium overload, in a similar manner to that mediated by ischemic preconditioning (20). We provide evidence that this inhibition involves mechanisms other than the translocation of PKC-ε to the mitochondria.

MATERIALS AND METHODS

Antibodies and chemicals. Polyclonal antibodies were raised in rabbits against purified rat heart mitochondrial whole adenine nucleotide translocase (ANT) and a COOH-terminal peptide of monocarboxylate transporter 1 (MCT1), conjugated to keyhole limpet hemocyanin, as described previously (32, 38). Anti-PKC-α and PKC-ε antibodies were purchased from Santa Cruz Biotechnology and anti-GAPDH antibody from Abcam. Phorbol ester (phorbol-12-myristate-13-acetate) was purchased from Sigma.

Animals. Male Sprague-Dawley rats were obtained from Charles River UK (Margate, UK) and received humane care in accordance with The Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 and under institutional license from the Government Home Office (London, UK).

Heart perfusion. This study conforms to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). The procedures were essentially the same as described previously (21, 23). Hearts (about 0.75 g) were removed from male Sprague-Dawley rats (250–260 g) and immediately arrested in ice-cold buffered Krebs-Henseleit solution. The aorta was rapidly cannulated, and the heart was perfused at 12 ml/min in the Langendorff mode using Krebs-Henseleit buffer containing (in mM) 118 NaCl, 25 NaHCO3, 4.8 KCl, 1.2 KH2PO4, 1.2 MgSO4, 11 glucose, and 1.2 CaCl2 gassed with 95% O2-5% CO2 at 37°C (pH 7.4). The monitoring of left ventricular developed pressure (LVDP) was performed with a water-filled balloon inserted into the left ventricle set to give an initial end-diastolic pressure of 2.5–5 mmHg. Hearts were perfused for 30 min in the presence or absence of 10 nM UCN, and, when required, global isothermic ischemia was then induced by halting perfusion and immersing the heart in perfusion buffer at 37°C. After 30 min ischemia, perfusion was restarted in the presence or absence of UCN and continued for the required time. Samples of perfuse were collected before ischemia and every 1 min during reperfusion for the spectrophotometric determination of lactate dehydrogenase (LDH) activity. At defined stages during the perfusion protocol, hearts were homogenized and mitochondria prepared as described in the following section.

Isolation of particulate and mitochondrial fractions. All procedures were carried out at 0–4°C. For measurement of MPTP opening and protein carboxylation, a mitochondrial fraction was prepared as follows. Ventricles were rapidly cut away, weighed, and homogenized with a Polytron homogenizer at setting 3 for 5 s in 5 ml of ice-cold sucrose buffer (in mM: 300 sucrose, 10 Tris·Cl, and 2 EGTA; pH 7.4) and buffer containing 5 mg/ml bovine serum albumin (BSA) added to a final volume of 40 ml. The homogenate was centrifuged for 2 min at 2,000 g to remove cell debris, and the supernatant was centrifuged at 10,000 g for 5 min. For studies of PKC translocation into crude particulate and purified mitochondrial fractions, all buffers contained protease inhibitors (Complete, Mini, EDTA-free protease inhibitor cocktail; Roche Diagnostics), and the homogenization and fractionation procedures were modified as follows. The initial homogenization was in 2 ml of sucrose buffer, and the homogenate was diluted to 6 ml with sucrose buffer containing BSA (5 mg/ml) before centrifugation at 2,000 g for 90 s. The resulting supernatant was centrifuged at 200,000 g for 45 min to produce a crude total particulate fraction. A small sample of the supernatant (cytosol) and the pellet were kept for analysis, whereas the remainder of the pellet was resuspended in 6 ml sucrose buffer containing 20% Percoll (wt/vol) and centrifuged at 12,000 g for 10 min to yield a purified mitochondrial pellet that was washed once in 6 ml sucrose buffer followed by centrifugation at 12,000 g.

Measurement of MPTP opening in vitro. The opening of the MPTP was determined at 25°C under energized and deenergized conditions by following the decrease in light scattering (monitored as A520 time course (17).

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Measurement of MPTP opening in situ using mitochondrial 2-[3H]deoxyglucose entrapment. Pretischemic loading of hearts was performed as previously described (21); when present, UCN was present at 10 nM throughout the perfusion protocol. Briefly, after a 15-min stabilization period, hearts were perfused in a recirculating mode with 40 ml of Krebs-Henseleit solution containing 0.5 mM 2-[3H]deoxyglucose (2-[3H]DG; 0.1 μCi/ml) for 30 min. Perfusion was then returned to flow through (noncirculating) mode with normal Krebs-Henseleit buffer. Following a further 15-min perfusion to wash
out extracellular 2-[3H]DG, the buffer flow was halted to initiate global isometric (37°C) ischemia. After 30 min ischemia, hearts were reperfused for 30 min in the presence or absence of 10 nM UCN before mitochondrial preparation and the determination of 2-[3H]DG and citrate synthase activity (21).

Protein carboxylation and PKC translocation assays. Protein carbonyls were analyzed according to Shacter and others as described previously (24, 41). Briefly, an aliquot of the mitochondrial proteins was derivatized with dinitrophenylhydrazine under acid denaturing conditions. Proteins were separated by SDS-PAGE and subject to Western blot analysis performed with anti-dinitrophenyl primary antibodies (Intergen) at 1:150 dilution. To correct for nonspecific binding of the antibodies, separate aliquots of the mitochondrial proteins that had been acid denatured but not treated with dinitrophenylhydrazine were run in parallel. PKC translocation following 30 min of total Langendorff perfusion was determined in cell fractions obtained from control and UCN-treated hearts as described in Heart perfusion. An additional group of hearts was perfused with Krebs-Henseleit buffer for 20 min and then with 200 nM phorbol ester for 10 min. Proteins were separated by SDS-PAGE and subject to Western blot analysis performed with antibodies against PKC-α, PKC-ε, ANT (a mitochondrial marker), and MCT1 (a specific plasma membrane marker). Blots were developed using anti-rabbit Ig horseradish peroxidase secondary antibody with enhanced chemiluminescence (ECL)/ECL+ detection (Amersham Biosciences). Each blot contained samples from control and UCN-treated hearts that had been perfused on the same day to allow direct comparison. Appropriate protein loading and exposures were used to ensure that band intensities were within the linear range. Quantification of blots was performed using an AlphaInnotech ChemiImager 4400 to image the blot, and band intensity was analyzed with AlphaEase v. 5.5 software.

Preparation of neonatal rat myocytes. Neonatal rat cardiac myocytes were prepared as previously described and cultured in gelatin-coated 24-well tissue culture plates in Dulbecco’s modified Eagle’s medium (DMEM) with fetal calf serum (15%) for 24 h before treatment (43). Most cells could be seen to beat spontaneously in a confluent monolayer 24–48 h after plating. After 24 h, the medium was replaced with DMEM supplemented with fetal calf serum (1%). Measurement of MPTP opening in neonatal cardiac myocytes using tetramethylrhodamine methyl ester fluorescent dyes. MPTP opening was assessed using tetramethylrhodamine methyl ester (TMRE), a cell-permeable, voltage-sensitive dye that accumulates in energized, but not depolarized, mitochondria. Plasma membrane rupture was revealed by staining cells with 12 g/ml 7-amino-actinomycin D (7-AAD), which permeates only damaged cells. Cytosfluorimetric analysis was performed using an Epics XL flow cytometer (Beckman Coulter) equipped with a 488-nm argon ion laser. The TMRE signal was analyzed in the FL2 channel, which was equipped with a bandpass filter at 575 ± 20 nm, and the 7-AAD signal was analyzed in the FL3 channel, which was equipped with a bandpass filter at 675 ± 20 nm. Data were acquired on a logarithmic scale. Arithmetic mean values of the median fluorescent intensities were determined for TMRE. Cell death was calculated as the percentage of cells positive for 7-AAD.

Preparation of adult rat cardiac myocytes. Adult rat myocytes were isolated by collagenase perfusion as previously described (19). Briefly, after anesthesia with pentobarbital sodium (55 mg/kg ip) and the administration of heparin sodium (300 IU), hearts were rapidly excised, placed in ice-cold buffer, and mounted on a nonrecirculating perfusion apparatus. All solutions used were based on a modified calcium-free Krebs-Ringer-HEPES (KRH) buffer containing (in mM) 116.0 NaCl, 5.4 KCl, 0.4 MgSO4, 2.0 HEPES, 0.9 Na2HPO4, and 10 glucose (pH 7.4). The perfusate was bubbled with 100% O2 and maintained at 37°C. The hearts were first perfused at 14 ml/min with KRH buffer. After 5 min, the hearts were perfused with KRH buffer containing 0.75 mg/ml collagenase (Worthington type II) and 44 µM CaCl2 for 10 min. Following perfusion, the hearts were removed from the perfusion apparatus, and the atria were trimmed away. The ventricles were minced and underwent several more digestions with collagenase. The cells were then washed with restoration buffer consisting of KRH buffer plus 10 mg/ml BSA and 44 µM CaCl2. The calcium concentration was gradually increased to 1.25 mM. After isolation, the cells were seeded onto sterilized laminin-coated 23-mm-diameter round coverslips and incubated for 60 min at 37°C in an atmosphere of 95% air-5% CO2 in medium 199 (M7653, Sigma) containing 1% penicillin-streptomycin (Sigma).

Culture of H9c2 cells. H9c2 cells were cultured in MEM containing 10% fetal calf serum and 1% penicillin-streptomycin in a humidified CO2 incubator.

Detection of reactive oxygen species production in cells. Cells were subjected to I/R or cultured in control buffer. Reperfusion medium contained 5 µM of acetylated 2’,7’-dichlorofluorescein diacetate (Molecular Probes). After 30 min, cells were trypsinized, stained with propidium iodide (PI), and analyzed by flow cytometry (Partec PAS). The average DCF signal was calculated from 5,000 live cells (i.e., which excluded PI).

Statistical analysis. Data are expressed as means ± SE, and the statistical difference between control and UCN samples was determined by Student’s t-test or ANOVA followed by Fisher protected least significant difference test for multiple comparisons. Differences were considered to be statistically significant when P < 0.05 (indicated by an asterisk) or P < 0.01 (indicated by double asterisks).

RESULTS

UCN improves recovery and survival of hearts following reperfusion and decreases MPTP opening. To examine whether UCN-mediated hemodynamic recovery (Fig. 1A) protects the heart by reducing the extent of opening of the MPTP, we employed the mitochondrial 2-[3H]DG entrapment technique, described previously (21). The results demonstrate that UCN treatment significantly reduces the extent of MPTP opening in the intact heart (Fig. 1B), consistent with its proposed role in necrotic cell death. The yield of mitochondria in these experiments, determined as the total citrate synthase activity of the mitochondrial pellet, was not altered by UCN treatment (Table 1).

In the same series of experiments, we further verified the acute protective effects of UCN administration by determining the hemodynamic performance of control Langendorff-perfused hearts and those treated with 10 nM UCN (Table 1). Before I/R, UCN exerted no significant effects on any parameter measured. However, UCN significantly reduced the time taken to initiate ischemic contracture (11.9 ± 0.6 vs. 7.6 ± 0.5 min, P < 0.01) although the time taken to reach maximal ischemic contracture (19.0 ± 0.7 vs. 18.4 ± 1.0 min) and its magnitude (32.1 ± 1.6 vs. 33.6 ± 1.6 mmHg) remained unaltered. Upon reperfusion, the recovery of LVDP was significantly greater in UCN-treated hearts (32.1 ± 7.1 vs. 64.6 ± 4.1 mmHg, P < 0.01), whereas the elevation of end-diastolic pressure was significantly reduced (48.1 ± 6.3 vs. 20.9 ± 4.0 mmHg) and there was no change in heart rate. This improvement in hemodynamic recovery shown by a restoration of LDVP (Fig. 1A) was associated with a significant decrease in LDH release, indicative of less necrotic cell death, and this was maintained for at least 30 min after the start of reperfusion (Fig. 1C).

Mitochondria isolated from UCN-treated hearts following I/R show less oxidative stress and are less sensitive to MPTP opening. Since the 2-[3H]DG entrapment technique showed that MPTP opening was decreased in UCN-treated hearts
Fig. 1. Urocortin (UCN) improves hemodynamic recovery of hearts following reperfusion and decreases mitochondrial permeability transition pore (MPTP) opening and lactate dehydrogenase (LDH) release. Langendorff-perfused rat hearts were preloaded with 2-[^H]deoxyglucose (2[^H]DG) and then subject to 30 min of ischemia followed by 30 min of reperfusion. Measurements were made of hemodynamic function (A), mitochondrial 2[^H]DG entrapment (B), and perfusate LDH (C) as described in MATERIALS AND METHODS. In hearts that were administered UCN, 10 nM UCN was present throughout the entire period of perfusion. Data are shown as means ± SE from for 8 separate hearts. Data shown were obtained from the experiments shown in Fig. 1 in which mitochondrial permeability opening was determined using the 2[^H]DG entrapment technique. The statistical significance of differences in the parameters caused by urocortin (UCN) treatment was calculated by Student’s *t*-test (*P < 0.01; †P < 0.05). LVDP, left ventricular developed pressure; EDP, end-diastolic pressure; IC, ischemic contracture.

Table 1. Effects of UCN on heart function

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<tr>
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<th>Preischemic</th>
<th>UCN</th>
<th>End-Ishemic</th>
<th>UCN</th>
<th>Reperfused</th>
<th>UCN</th>
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<tr>
<td></td>
<td>Control</td>
<td></td>
<td>Control</td>
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<td>Control</td>
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<tr>
<td>LVDP, mmHg</td>
<td>90.0±1.8</td>
<td>91.6±3.1</td>
<td>—</td>
<td>—</td>
<td>32.1±7.1</td>
<td>64.6±4.1*</td>
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<tr>
<td>EDP, mmHg</td>
<td>3.4±0.4</td>
<td>3.6±0.5</td>
<td>—</td>
<td>—</td>
<td>48.1±6.3</td>
<td>20.9±4.0*</td>
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<tr>
<td>Heart rate, beats/min</td>
<td>307.5±7.6</td>
<td>296.3±10.6</td>
<td>—</td>
<td>—</td>
<td>315.0±9.9</td>
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<tr>
<td>Aortic pressure, mmHg</td>
<td>86.6±3.8</td>
<td>78.8±2.3</td>
<td>—</td>
<td>—</td>
<td>107.5±6.6</td>
<td>86.4±2.2†</td>
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<tr>
<td>Time to IC, min</td>
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<td>—</td>
<td>11.9±0.6</td>
<td>7.6±0.5*</td>
<td>—</td>
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<tr>
<td>Time to maximal IC, min</td>
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<td>—</td>
<td>19.0±0.7</td>
<td>18.4±1.0</td>
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<td>Maximal IC, mmHg</td>
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<td>32.1±1.6</td>
<td>33.6±1.6</td>
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<tr>
<td>Citrate synthase recovery, U/g heart wet wt</td>
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<td>—</td>
<td>1.87±0.2</td>
<td>1.77±0.2</td>
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Data are means ± SE from for 8 separate hearts. Data shown were obtained from the experiments shown in Fig. 1 in which mitochondrial permeability transition pore opening was determined using the 2[^H]DG entrapment technique. The statistical significance of differences in the parameters caused by urocortin (UCN) treatment was calculated by Student’s *t*-test (*P < 0.01; †P < 0.05). LVDP, left ventricular developed pressure; EDP, end-diastolic pressure; IC, ischemic contracture.
whether UCN treatment might do the same by performing rapid subcellular fractionation on control and UCN-treated hearts, followed by SDS-PAGE and Western blot analysis. We devised a protocol (see MATERIALS AND METHODS) in which the crude homogenate was first clarified of cell debris by a low-speed centrifugation and then a cytosolic fraction and crude particulate fraction (mitochondria plus sarcoplasmic reticulum, microsomes, and plasma membranes) obtained by a centrifugation at 200,000 g. A purified mitochondrial fraction was obtained from the particulate fraction by Percoll gradient centrifugation, and all samples were analyzed by SDS-PAGE and Western blot analysis for PKC-α/H9251, PKC-ε, the ANT (to determine mitochondrial enrichment), and MCT1 (to monitor plasma membrane contamination). Data are shown in Fig. 3. In Fig. 3A, we show that the crude particulate fraction contained both MCT1 and ANT as expected. Following Percoll gradient centrifugation, ANT was enriched in the resulting mitochondrial fraction, but all MCT1 was lost, confirming the loss of plasma membrane contamination. Little or no PKC-α or PKC-ε was found in the mitochondrial fraction, and there was no evidence of any increase following UCN treatment. Furthermore, although both PKC isoforms could be detected in the crude particulate fraction, there was again no evidence for an increase following UCN treatment. By contrast, when hearts were treated with phorbol-12-myristate-13-acetate (200 nM) for 10 min and the same fractionation procedure was employed, there was a clear increase in the amount of PKC-α and PKC-ε in both the crude particulate and purified mitochondrial fractions, whereas the cytosolic levels of both decreased. The mean results of six experiments are reported in Fig. 3B. Data are presented as the ratio of PKC-α/H9251 or PKC-ε in the crude particulate and mitochondrial fractions to that in the cytosolic fraction to provide the most sensitive indicator of translocation.

We also verified that UCN pretreatment had no effect on the translocation of PKC-ε to the mitochondrial fraction after 30 min of ischemia followed by 3 min of reperfusion (Fig. 4, A and B). Furthermore, the amount of PKC-δ detected in these extracts did not vary between control and I/R samples (data not shown). These data confirm that UCN had no detectable effect on PKC translocation in the perfused heart, whereas phorbol ester had a profound effect.

UCN treatment protects neonatal cardiac myocytes against simulated I/R. There are two windows of preconditioning: the early phase that occurs immediately (acute) after the preconditioning stimulus and then declines after an hour or so, and a second (chronic) window emerging about 24 h later (48). We wished to assess whether both acute and chronic treatment with UCN protect cardiac myocytes at the level of the MPTP.
overnight recovery in normal tissue culture medium (Fig. 5) followed by an overnight incubation in simulated ischemic medium followed by an incubation in normal control buffer for 60 min before I/R, which entailed an incubation period of 4 h in simulated ischemic buffer followed by a further overnight incubation in normal culture medium (Fig. 5, A and B). All samples were treated with 7-AAD and analyzed by flow cytometry to assess cell viability where percent cell death is shown from 5,000 separate events. I/R increased the proportion of dead cells to >70%. No effects of either UCN or phorbol ester were observed on the amount of monocarboxylate transporter 1 (MCT1) and adenine nucleotide translocase (ANT) in each fraction (data not shown). Cyt, cytosolic fraction; Prt, crude particulate fraction; Mit, Percoll-purified mitochondrial fraction.

Chronic treatments cannot be investigated in the Langendorff-perfused heart, but primary cultured cardiac myocytes subject to simulated I/R might provide an appropriate model. For instance, using a trypan blue assay of cell death, we have previously demonstrated that UCN treatments of between 30 min and 24 h protect neonatal cardiac myocytes (6). Here we have first examined whether flow cytometry is effective at detecting protection against cell death in this model. Neonatal rat cardiac myocytes were treated with 10 nM UCN or a control buffer or treated overnight (16 h) before 4 h in simulated ischemic buffer followed by a further overnight incubation in normal culture medium (Fig. 5, C and D). All samples were treated with 7-AAD and analyzed by flow cytometry to assess cell viability where percent cell death is shown from 5,000 separate events. I/R increased the proportion of dead cells to >70%, and acute pretreatment with UCN significantly reduced this percentage to 37 ± 7.5% (P < 0.01). Accordingly, a chronic pretreatment with UCN also offered protection by reducing cell death by >30%, thereby establishing that both acute and chronic treatment with UCN protects adult cardiac myocytes from I/R injury. As described for the neonatal experiments, adult cardiac myocytes were either acutely pretreated with 10 nM UCN or a control buffer or treated overnight (16 h) before 4 h in simulated ischemic buffer followed by a further overnight incubation in normal culture medium (Fig. 5, A and B). All samples were treated with 7-AAD and analyzed by flow cytometry to assess cell viability where percent cell death is shown from 5,000 separate events. I/R increased the proportion of dead cells to >70%. No effects of either UCN or phorbol ester were observed on the amount of monocarboxylate transporter 1 (MCT1) and adenine nucleotide translocase (ANT) in each fraction (data not shown). Cyt, cytosolic fraction; Prt, crude particulate fraction; Mit, Percoll-purified mitochondrial fraction.

Fig. 3. Acute UCN treatment of perfused hearts does not lead to PKC translocation to the mitochondria. Hearts were perfused in the presence or absence of 10 nM UCN for 30 min or with 200 nM phorbol ester (phorbol-12-myristate-13-acetate) for 10 min after 20 min of control perfusion. Hearts were then rapidly homogenized and subject to rapid subcellular fractionation as described in MATERIALS AND METHODS. Proteins in each fraction were analyzed by SDS-PAGE and Western blot analysis with the antibodies indicated. For each experimental condition, control samples were run on the same gel to allow direct comparison. Representative blots (A) and quantitative data (B) from 6 separate experiments are shown. This was obtained by scanning the blots and expressing the amount of particulate and mitochondrial PKC-α and PKC-ε as ratios to the amount in the cytosol of the same heart. Data are shown as means ± SE of 6 separate experiments (*P < 0.05 for phorbol ester vs. control). No effects of either UCN or phorbol ester were observed on the amount of monocarboxylate transporter 1 (MCT1) and adenine nucleotide translocase (ANT) in each fraction (data not shown). Cyt, cytosolic fraction; Prt, crude particulate fraction; Mit, Percoll-purified mitochondrial fraction.

Fig. 4. Acute UCN treatment of perfused hearts does not lead to PKC-ε or PKC-δ translocation to the mitochondria (Mit) upon reperfusion. After 5 min of control perfusion, hearts were perfused in the presence or absence of 10 nM UCN for 30 min before 30 min of global ischemia. They were then reperfused for 3 min in the presence or absence of 10 nM UCN before rapid homogenization and subcellular fractionation as described in MATERIALS AND METHODS. Proteins in each fraction were analyzed by SDS-PAGE and Western blot analysis with the antibodies indicated. For each experimental condition, control samples were run on the same gel to allow direct comparison. Representative blots (A) are shown, and quantitative data (means ± SE, 6 separate experiments; B) were obtained as described in Fig. 3. No effects of either UCN or phorbol ester were observed on the amount of MCT1 and ANT in each fraction (data not shown).
Fig. 5. UCN treatment of isolated neonatal and adult cardiac myocytes protects them from simulated I/R. Primary neonatal (A and B) or adult (C and D) rat cardiac myocytes were treated with 10 nM UCN or control buffer for 60 min (A and C) or 16 h (B and D) before simulated I/R (+ve). Simulated ischemia was achieved by replacing culture medium with ischemia buffer and placing the cells in an ischemic chamber for 4 h. After ischemia, the medium was replaced and cells were cultured for a further 16 h in normal culture conditions to simulate reperfusion. All samples were treated with the vital dye 7-amino-actinomysin D (7-AAD; 12 μg/ml) immediately before flow cytometry. The percent cell death was determined from 5,000 separate events (*P < 0.05 and **P < 0.01 compared with relevant controls).

UCN treatment inhibits MPTP opening in neonatal cardiac myocytes subject to simulated I/R. Primary rat neonatal cardiac myocytes were subjected to simulated I/R with or without a 16-h pretreatment of 10 nM UCN. Some wells were treated for 16 h before I/R with 0.2 μM CsA, a compound known to inhibit the opening of the MPTP (10). Before I/R, cells were preloaded with 200 nM TMRM for assessment of mitochondrial depolarization. The cells were exposed to 4 h of simulated ischemia after which the medium was replaced and the cells were cultured for a further 16 h in normal culture conditions to simulate reperfusion. The mean fluorescence intensity of TMRM was determined from 5,000 separate events using flow cytometry. As demonstrated (Fig. 6A), treatment with a mitochondrial uncoupler (mitochondrial carbonyl cyanide m-chlorophenylhydrazone) reduced TMRM fluorescence to background (“unstained”) levels, confirming that TMRM fluorescence could be used to detect mitochondrial depolarization. Following I/R, fluorescence was also substantially reduced as predicted to occur after the opening of the MPTP. Confirmation that this depolarization was a consequence of MPTP opening was provided by CsA, a known inhibitor of MPTP opening, which largely prevented the drop in fluorescence caused by I/R (Fig. 6A). Chronic pretreatment with UCN also prevented a drop in fluorescence in response to I/R (Fig. 6A), suggesting that UCN protected the MPTP from opening in response to I/R. Further evidence that UCN acts at the level of the MPTP was provided by the observation that coinubcation with CsA did not provide any additional protection against loss of mitochondrial potential (Fig. 6A).

UCN treatment inhibits MPTP opening in adult cardiac myocytes subject to simulated I/R. To determine whether UCN can also protect adult cardiac myocytes at the level of the MPTP, similar experiments were performed using primary adult rat cardiac myocytes either treated chronically (16 h) or acutely (1 h) with 10 nM UCN (Fig. 6, B and C, respectively). Before I/R, cells were preloaded with 200 nM TMRM for assessment of mitochondrial permeability. Cells were then exposed to 4 h of simulated ischemia, before media was replaced, and cells were cultured for a further 16 h in normal culture conditions to simulate reperfusion. Whereas simulated I/R caused a decrease in the average fluorescence intensity, short- or long-term UCN pretreatment largely prevented this decrease (Fig. 6, B and C).

These experiments demonstrate that chronic, as well as acute, pretreatment with UCN can protect both neonatal and adult rat cardiac myocytes from simulated I/R by preventing the opening of the MPTP and hence preserving the mitochondrial membrane potential.

UCN treatment reduces the level of oxidative stress during reperfusion via activation of PKC. In Mitochondria isolated from UCN-treated hearts following I/R show less oxidative stress and are less sensitive to MPTP opening, we measured less carbonylated protein in mitochondria isolated from UCN-treated hearts after reperfusion. To support our hypothesis that this is due to UCN preventing an increase in oxidative stress at reperfusion, we directly measured reactive oxygen species (ROS) production in isolated cells exposed to I/R by flow cytometry of cells incubated with DCF, a cell permeable fluorescent probe. During 30 min of postischemic reperfusion, the average cell fluorescence increased significantly in neonatal cardiac myocytes (Fig. 7A). Overnight or 30-min pretreatment with 10 nM UCN reduced postischemic ROS production to background levels. The rodlike shape and inherent fragility make adult cardiac myocytes difficult to analyze by flow cytometry, and data using these cells were unreliable. However, we verified the above results showing that UCN can reduce the levels of ROS at reperfusion using H9c2 cells, a cell line derived from cardiac myocytes (Fig. 7B). The ability of UCN to reduce oxidative stress at reperfusion depends on the activation of PKC, since treatment of H9c2 cells with chelerythrine, an inhibitor of PKC, completely abolished the effect of UCN (Fig. 8).
DISCUSSION

UCN treatment inhibits MPTP opening. The data presented here show that the protection against reperfusion injury provided by UCN pretreatment of either the isolated perfused rat heart or primary adult and neonatal cardiac myocytes is associated with inhibition of the MPTP. Thus, in the perfused heart, the enhanced recovery of hemodynamic function of the perfused heart mediated by UCN treatment (Table 1 and Fig. 1A) and the decreased necrotic damage determined by the release of LDH (Fig. 1C) were accompanied by less MPTP opening detected in situ by the 2-[3H]DG entrapment technique (Fig. 1B). Furthermore, we show that following I/R, mitochondria isolated from the UCN-treated hearts were less sensitive to MPTP opening in vitro, confirming that following UCN treatment, conditions at reperfusion do favor less MPTP opening (Fig. 2A). Similarly, in isolated neonatal (Fig. 6A) and adult cardiac myocytes (Fig. 6, B and C), measurement of mitochondrial membrane potential confirmed that UCN pretreatment reduced MPTP opening following simulated I/R. This was accompanied by less cell death (Fig. 5). In both cultured neonatal and adult cardiac myocytes, it was also possible to demonstrate that chronic UCN treatment inhibited MPTP opening (Fig. 6, A and B).

The protection conferred by UCN was not additive with that of CsA, a compound known to prevent MPTP opening, which therefore suggested that UCN treatment prevented MPTP opening. Although it is known that CsA can also inhibit calcineurin, the concentrations used here are optimal for preferential inhibition of MPTP (13, 34). Furthermore, several studies have shown that CsA analogs which do not inhibit calcineurin are also cardioprotective (1, 12, 14). Thus, when these data are considered in combination with data from Figs. 1B and 2A, which directly demonstrate that UCN reduces MPTP opening in the isolated heart and in isolated cardiac mitochondria, respectively, it seems likely that the MPTP plays a central role in UCN-mediated protection.

The mechanism by which UCN inhibits MPTP opening. The signaling pathways through which UCN exerts its effects may involve ERK1/2 and PKC-ε since activation of both have been reported in isolated cardiac myocytes and the isolated perfused rat hearts (5, 6, 29, 40). In this respect, UCN would appear similar to many other cardioprotective agents since it has been proposed that all cardioprotective regimes function via activation of either or both the ERK1/2 or phosphatidylinositol 3-kinase/Akt pathways with PKC-ε playing either an upstream or downstream role (18, 33, 48). Exactly how these pathways lead to an inhibition of the MPTP with a consequent protection of the heart remains unclear. One possibility is that molecules, such as nitric oxide that is downstream of cardioprotective pathways, directly affect the susceptibility of the MPTP to calcium-induced mitochondrial swelling, as suggested by Wang et al. (46). However, our data are not compatible with a
The effect of phosphorylation by PKC-ε or after ischemia (Figs. 3 and 4). These data imply that a direct fraction under control or UCN-treated conditions, either before PKC-ε, which has been proposed to promote mitochondrial/H9254 fraction was small (Fig. 3). Although a substantial level of amount of these PKC isoforms in the pure mitochondrial component is unlikely. Even after phorbol ester treatment, the change in sensitivity to MPTP opening in vitro (Fig. 2) or the H9c2 cardiac myocyte cell line (Fig. 7). UCN treatment of cultured cells results in less reactive oxygen species (ROS) production during simulated reperfusion after ischemia. Primary neonatal cardiac myocytes (A) or the H9c2 cardiac myocyte cell line (B) were subjected to simulated I/R after overnight (UCN o/n) or 30-min pretreatment (UCN 30’) with 10 nM UCN. At reperfusion, cells were transferred to medium containing dichlorofluorescein (DCF) for 30 min before analysis by flow cytometry, as described in MATERIALS AND METHODS. The increase in mean fluorescence is indicated compared with control (Con) cultures ± UCN. Data are averaged over 3 independent experiments (*P < 0.05 compared with the increase due to ischemia).

model in which acute treatment with UCN involves a modification of components of the MPTP, since mitochondria isolated immediately after UCN treatment and before I/R show no change in sensitivity to MPTP opening in vitro (Fig. 2A). Furthermore, in our perfused heart model, when all plasma membrane contamination was removed by Percoll treatment, almost no PKC-ε or PKC-α was detected in the mitochondrial fraction under control or UCN-treated conditions, either before or after ischemia (Figs. 3 and 4). These data imply that a direct effect of phosphorylation by PKC-ε or PKC-α of any MPTP component is unlikely. Even after phorbol ester treatment, the amount of these PKC isoforms in the pure mitochondrial fraction was small (Fig. 3). Although a substantial level of PKC-δ, which has been proposed to promote mitochondrial damage (9), was found in the mitochondrial fraction after I/R as described by others (8), this, too, was unaltered by UCN treatment (Fig. 4).

The present data may appear to be at odds with our earlier data where we used immunofluorescence confocal microscopy and Western blot analysis to demonstrate UCN-mediated translocation of PKC-ε to the mitochondria in neonatal cardiac myocytes and to the membrane fraction of the perfused heart and cultured cardiac myocytes (29). However, in these earlier experiments, unlike in our current studies, we did not isolate purified mitochondria for the Western blot analysis experiments. Furthermore, the colocalization of PKC-ε with Mitotracker Green following UCN treatment in the earlier studies was only partial. Indeed, there were many mitochondria that had little associated PKC-ε, and we cannot rule out that, even in those mitochondria with PKC-ε, it was not actually bound to other membranes in very close proximity to the mitochondria. Whatever the explanation, we conclude that PKC-ε translocation to the mitochondria is not essential for it to exert its inhibitory effect on MPTP opening.

This conclusion does not imply that PKC-ε plays no role in the action of UCN, rather that its effect on the MPTP is mediated indirectly. A plausible explanation for this would be a decrease in ROS production at reperfusion mediated by UCN. This is suggested by the decrease in oxidative stress experienced by the mitochondria as reflected in the lower protein carbonylation (Fig. 2B). These data are similar to those reported for ischemic preconditioning, where we have also argued that protection is mediated indirectly by decreasing ROS and possibly calcium overload at reperfusion (20, 24). We also showed that the level of ROS production by isolated cells during reperfusion, detected using a fluorescent substrate, is decreased by pretreatment with UCN (Fig. 7). This action of UCN requires the activity of PKC (Fig. 8), further supporting the hypothesis that PKC-mediated cardioprotection proceeds indirectly, via preventing ROS. ROS are known to oxidize mitochondrial proteins, as revealed by our protein carbonylation data (Fig. 2B), and such oxidative modification of a component of the MPTP (most likely the ANT and possibly its closely associated cardiolipin) is now thought to be the most critical factor in MPTP opening (15, 16, 25). Thus reduction in ROS by UCN provides a powerful mechanism to inhibit MPTP opening and so protect the heart. However, it cannot be ignored that ROS production on reperfusion could also be secondary to MPTP opening and thus the ability of UCN to decrease oxidative damage to the mitochondria might be a consequence of reduced MPTP opening rather than a cause (4, 49). Either way, the lack of an additive effect of CsA, a compound known to act on the MPTP, on mitochondrial depolarization in isolated cardiac myocytes suggests that inhibition of MPTP opening at reperfusion is a critical factor in the protection exerted by UCN.
Our data conclusively demonstrate that UCN treatment protects the heart from I/R by inhibiting the opening of the MPTP at reperfusion. In this regard they confirm the critical role of MPTP in mediating reperfusion injury and its importance as a target for cardioprotection. Our data also provide further evidence that the focus of our attention in understanding the mechanism of preconditioning strategies should be upstream of the mitochondria. In particular, it might be prudent to investigate the role of PKC-ε in decreasing both calcium overload and oxidative stress at reperfusion since these may well be responsible for the inhibition of MPTP opening.

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REFERENCES


