KATP Channel Gene Expression Is Induced by Urocortin and Mediates Its Cardioprotective Effect

K.M. Lawrence, A. Chanalaris, T. Scarabelli, M. Hubank, E. Pasini, P.A. Townsend, L. Comini, R. Ferrari, A. Tinker, A. Stephanou, R.A. Knight and D.S. Latchman

Circulation 2002;106;1556-1562; originally published online Aug 26, 2002;
DOI: 10.1161/01.CIR.0000028424.02525.AE
Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7322. Online ISSN: 1524-4539

K_{ATP} Channel Gene Expression Is Induced by Urocortin and Mediates Its Cardioprotective Effect

K.M. Lawrence, PhD; A. Chanalaris, MSc; T. Scarabelli, MD; M. Hubank, PhD; E. Pasini, MD; P.A. Townsend, PhD; L. Comini, MSc; R. Ferrari, MD; A. Tinker, PhD; A. Stephanou, PhD; R.A. Knight, MD, PhD; D.S. Latchman, PhD, DSc

Background—Urocortin is a novel cardioprotective agent that can protect cardiac myocytes from the damaging effects of ischemia/reperfusion both in culture and in the intact heart and is effective when given at reperfusion.

Methods and Results—We have analyzed global changes in gene expression in cardiac myocytes after urocortin treatment using gene chip technology. We report that urocortin specifically induces enhanced expression of the Kir 6.1 cardiac potassium channel subunit. On the basis of this finding, we showed that the cardioprotective effect of urocortin both in isolated cardiac cells and in the intact heart is specifically blocked by both generalized and mitochondrial-specific K_{ATP} channel blockers, whereas the cardioprotective effect of cardiotrophin-1 is unaffected. Conversely, inhibiting the Kir 6.1 channel subunit greatly enhances cardiac cell death after ischemia.

Conclusions—This is, to our knowledge, the first report of the altered expression of a K_{ATP} channel subunit induced by a cardioprotective agent and demonstrates that K_{ATP} channel opening is essential for the effect of this novel cardioprotective agent. **(*Circulation*. 2002;106:1556-1562.)**

Key Words: potassium channel ■ urocortin ■ ischemia ■ reperfusion

Urocortin (Ucn) is a member of the corticotrophin-releasing hormone (CRH) family of peptides and was originally identified in the rat brain.[1,2] Mature Ucn like CRH is derived from a larger propeptide and comprises 40 amino acids, which share 45% identity to mature CRH.[2] We have shown previously that Ucn is also expressed in the heart and that the abundance of the Ucn mRNA in cultured cardiac myocytes is increased by exposure to heat shock or simulated ischemia.[3] Moreover, Ucn peptide is released after exposure of cultured cardiac myocytes to simulated ischemia in vitro.[3]

We have previously demonstrated that exogenous Ucn protects cardiac myocytes from cell death after exposure to simulated ischemia/reperfusion (I/R) in vitro.[4,5] Moreover, Ucn has a similar effect in the isolated perfused heart exposed to ischemia ex vivo[5] and is also protective when administered to the heart in the intact animal (T.M. Scarabelli, MD, unpublished data, 2002). Most importantly, both in cultured cardiac cells and in the intact heart, Ucn has a clear protective effect when administered after ischemia at the time of reperfusion as well as when it is added before ischemia.[5]

It has previously been shown that protection by Ucn is dependent on its ability to activate the p42/p44 mitogen-activated protein kinase (MAPK) pathway.[5] Interestingly, the protective effect of the interleukin-6–like cytokine cardiotrophin-1 (CT-1) is also dependent on the activation of this pathway.[6,7] However, despite this similarity the protective effect of CT-1 does not require de novo protein synthesis,[7] whereas inhibition of de novo protein synthesis with cycloheximide prevents the protective effect of Ucn.[8]

In view of the lack of knowledge concerning the targets by which Ucn mediates its cardioprotective effect and the requirement for de novo protein synthesis, we have used Affymetrix gene chip technology to identify the global changes in gene expression that occur in cardiomyocytes given a protective treatment with Ucn.

Methods

Rat Neonatal Cardiac Myocyte Preparation

Hearts were removed from 2-day-old Sprague-Dawley rats, and ventricular myocytes were isolated, cultured, and transfected using the calcium phosphate method as previously described.[4,5,7] For the gene chip experiments, cells were either treated with 10^{-8} mol/L Ucn for 24 hours or untreated. This dose and timing of Ucn has previously been shown to be cardioprotective.[4,5]

Affymetrix Gene Chip Analysis

First-Strand cDNA Synthesis

RNA was isolated using the RNA isolator solution TRIZoL (Gibco BRL). Five micrograms of total RNA derived from myocytes left

Received May 1, 2002; revision received June 16, 2002; accepted June 16, 2002.

From the Medical Molecular Biology Unit (K.M.L., A.C., T.S., M.H., A.S., D.S.L.), Institute of Child Health, University College London, London, England; Cardiovascular Pathophysiology Research Centre (E.P., L.C., R.F.), S. Maugeri Foundation IRCCS, University of Ferrara, Italy; Centre for Clinical Pharmacology (A.T.), Department of Medicine, University College London, England; and National Heart and Lung Institute (R.A.K.), Royal Brompton Hospital, London, England.

Correspondence to D.S. Latchman, PhD, DSc, Medical Molecular Biology Unit, Institute of Child Health, University College London, 30 Guilford Street, London WC1N 1EH, UK. E-mail d.latchman@ich.ucl.ac.uk

Circulation **is available at http://www.circulationaha.org** **DOI: 10.1161/01.CIR.0000028424.02525.AE**

untreated or treated with Ucn were independently incubated with an oligoT(24) primer containing a 3′ T7 RNA polymerase promoter sequence and reverse transcribed using Superscript II reverse transcriptase (Gibco BRL).

Second-Strand Synthesis

First-strand cDNA was added to a second-strand synthesis reaction containing DNA polymerase I, RNAse H, and *Escherichia coli* DNA ligase and incubated for 2 hours at 16°C. After this reaction, double-stranded DNA was polished using T4 DNA polymerase and then extracted from the reaction mixture using phenol/chloroform and precipitated. The DNA pellet was resuspended in diethylpyrocarbonate-treated water.

Labeled Antisense cRNA Target

Double-stranded DNA was in vitro transcribed with T7 RNA polymerase in the presence of biotinylated nucleotides. The reaction was carried out for 5 hours at 37°C. Biotinylated cRNA was fragmented into ≈100 bases in length. cRNA from control or Ucn-treated myocytes were hybridized to two rat genome U34A chips (Affymetrix) at 45°C overnight.

Washing and Staining

Rat gene chips were washed several times in nonstringent and stringent wash buffer according to the Affymetrix fluidics station protocol. Phycoerythrin linked to streptavidin was used to label the hybridized target. The chips were scanned two times each to give an accurate value for the laser-induced excitation fluorescence, which is a measure of transcript abundance.

Data Analysis

Affymetrix software was used to generate a comparison of the two chips. Data could then be presented as a list of positive and negative fold changes in transcript levels.

Polymerase Chain Reaction

The following primers were used to semiquantify transcript levels of several genes of interest derived from the gene chip data: Kir 6.1, forward primer, 5′-GCTTCGTGTCGATTGTGACTG-3′, and reverse primer, 5′-TTCCTCCGGATGGAGTTGCTC-3′; Kir 6.2, forward primer, 5′-ATGATCATTAGCGCCACCAT-3′, and reverse primer, 5′-GTGGGCACTTTAACGGTGTT-3′; SUR2, forward primer, 5′-GGTTGTGGCAAATCGTCT-3′, and reverse primer, 5′-GGAGAATGGATCGTCCAA-3′; and rat cyclophilin, forward primer 5′-CGAGCTGTTTGCAGACAAAG-3′, and reverse primer 5′-TTCTTGCTGGTCTTGCCATT-3′

Western Blots

Western blotting was carried out following standard protocols. Anti-Kir6.1 antibody was kindly provided by Dr A. Tinker. Anti-SUR1 was a kind gift of Dr Rao Sivaprasadaro, University of Leeds, UK. Anti-actin antibody was purchased from Santa Cruz.

Animal Model

Experiments in isolated rat hearts were carried out as previously described.[9]

Statistics

ANCOVA, with time as the covariate, and post hoc analyses were used to test the principal component with contrast. The Bonferroni correction was then applied and probability values <0.05 were considered significant.

Results

Screening of Affymetrix Gene Chip Array

To analyze the effects of Ucn on gene expression, neonatal rat cardiac myocytes were treated for 24 hours with Ucn (which is sufficient to produce a protective effect of Ucn)[5] and compared with similarly cultured untreated cardiac myocytes by screening Affymetrix gene chip microarrays containing 7000 rat genes.

Changes in Gene Expression Produced by Urocortin Treatment

Gene	Fold Change
Cardiac-specific sodium channel	+8
Interleukin-1 receptor–related protein	+5
Platelet-derived growth factor	+4.5
Protein kinase C ϵ	+3.4
Kir 6.1	+2.6
Growth hormone	+2.5
VCAM1	+2.1
Protein kinase N	+2
Bcl2	+2
Cytochrome P_{450}	−7.4
Cytochrome oxidase	−4
Nicotinic Ach receptor α subunit	−3.4
Bcl2 β	−2.5
Calcium-independent phospholipase A_2	−2.5
Kir 6.2	NC
SUR 2	NC

NC indicates no change in transcript level; +, fold increase in the Ucn-treated sample compared with control; and −, fold decrease in the Ucn-treated sample compared with control.

The great majority of genes showed no significant difference in expression between the two samples. However, several genes showed increases of 2-fold or more in the Ucn-treated sample compared with the untreated sample whereas several others showed decreases of 2-fold or more (Table). Hence, Ucn does indeed induce significant changes in gene expression in cultured neonatal cardiac myocytes.

Although each of the changes in gene expression that we detected is of potential interest, we focused initially on the increased expression of the ATP-sensitive inwardly rectifying potassium channel Kir 6.1, which showed a 2.6-fold increase in expression in the Ucn-treated sample. This gene was chosen because a number of experiments with pharmacological compounds that either open or block K_{ATP} channels have suggested that their opening has cardioprotective effects against ischemia and is likely to be involved in the cardioprotective effect of preconditioning by repeated short bursts of ischemia or treatment with adenosine.[10–13] Moreover, the effect on the Kir 6.1 channel appeared to be a specific one, given that the other member of the Kir 6.X inwardly rectifying potassium channel subfamily, Kir 6.2, was also present on the gene chip and showed no change in gene expression (Table). Similarly, the sulfonylurea receptor SUR2 that associates with the Kir 6.X channel to form a functional K_{ATP} channel showed no change in gene expression (Table).

Enhanced Expression of the Kir 6.1 mRNA and Protein After Ucn Treatment

To confirm the change in gene expression of the Kir 6.1 channel, we prepared further samples of mRNA from untreated and Ucn-treated cardiac myocytes and measured the change in Kir 6.1 mRNA both by a reverse transcriptase/

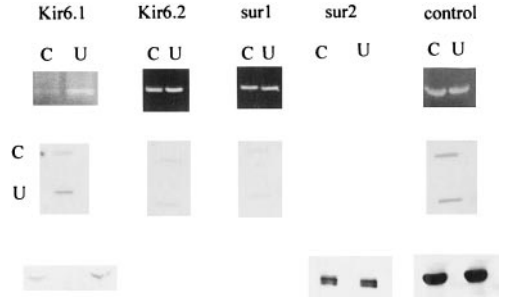

Figure 1. Reverse transcriptase/polymerase chain reaction (top panel), RNA slot blot (middle panel), or Western blots (lower panel) of Kir 6.1, Kir 6.2, SUR1 and SUR2 RNA, and protein levels derived from neonatal primary cardiomyocytes treated for 24 hours with Ucn (U) and untreated controls (C). Cyclophilin was used as an internal control for the RNA analysis and actin for Western blots.

polymerase chain reaction assay that we have previously used to quantify different mRNAs in limited amounts of material[14] and also by a slot-blot assay using a Kir 6.1–specific probe. The results of this experiment (Figure 1) confirmed the increase in Kir 6.1 RNA levels in the Ucn-treated sample. This increase was specific for Kir 6.1 with no increase in Kir 6.2 or SUR 2 being observed in the Ucn-treated sample (Figure 1). Interestingly, a small induction of Kir 6.1 was observed on treatment of the cells with the K_{ATP} channel opener cromakalim (data not shown), but this was smaller than that observed with Ucn.

We also prepared protein extracts from cardiac myocytes treated with Ucn or left untreated and probed these in a Western blot analysis with a specific antibody to Kir 6.1. A clear increase was observed in the protein abundance of Kir 6.1 in the Ucn-treated sample compared with the control sample, whereas no increase was observed in the Ucn sample for the SUR 1 sulfonylurea receptor or for the control actin protein (Figure 1). Hence, Ucn causes a specific increase in the mRNA and protein for Kir 6.1 while not affecting the closely related Kir 6.2 channel or the sulfonylurea receptors that associate with the Kir 6.1 and 6.2 channels.

Kir 6.1 Is Induced by I/R But Not by CT-1

In a time course study (Figure 2), we observed maximal induction of Kir 6.1 protein expression after 24 hours of Ucn treatment, although clearly detectable induction was also observed at 8 or 16 hours of Ucn treatment. Induction of Kir 6.1 expression was not a general effect of treatment with cardioprotective agents, given that CT-1 did not induce Kir 6.1 expression (Figure 2), even though like Ucn, it induces cardioprotection in a p42/p44 MAPK-dependent manner.[6,7]

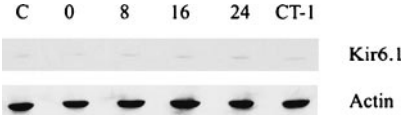

Figure 2. Time course of induction of Kir 6.1 protein, as determined by Western blotting of extracts from neonatal primary cardiomyocytes exposed to Ucn for the indicated period (in hours). The effect of 24-hour treatment with CT-1 is shown for comparison.

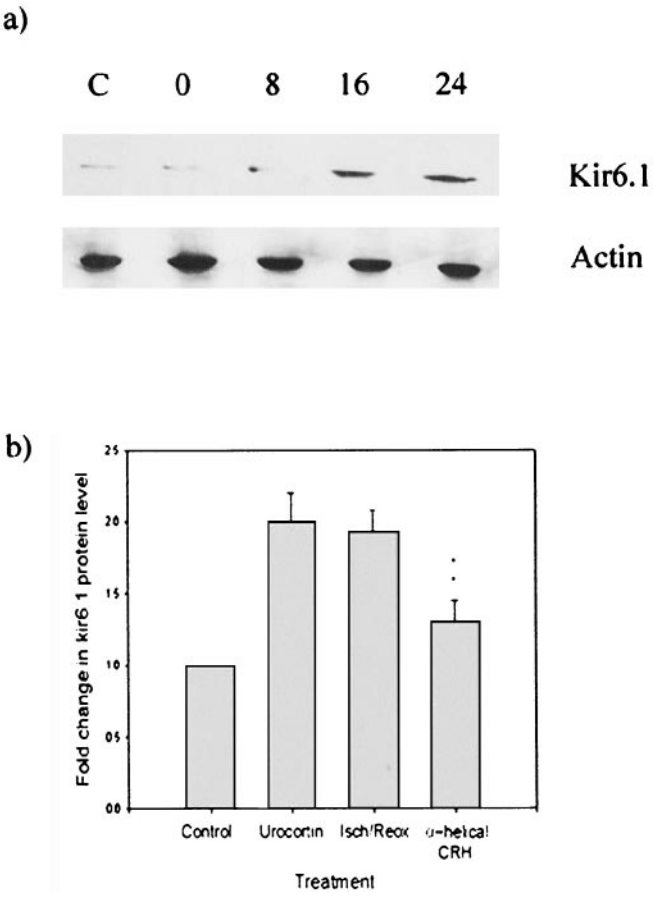

Figure 3. a, Kir 6.1 protein levels in neonatal cardiomyocytes exposed to 4 hours of simulated ischemia followed by the indicated period (in hours) of reoxygenation. b, Effect on induction of Kir 6.1 by ischemia reoxygenation of including the Ucn antagonist α-helical CRH in culture medium. Values are means of three independent experiments; bars, SD. $^{**}P<0.01$ ischemia/reoxygenation in the absence vs presence of α-helical CRH. C indicates control.

It has previously been reported that Kir 6.1 expression is also induced by exposure of the intact heart to I/R.[15] By exposing cultured cardiac cells to 4 hours of simulated ischemia followed by varying periods of reoxygenation, we confirmed that this effect could also be observed in culture with induction of Kir 6.1 being maximal at 16 or 24 hours of reperfusion (Figure 3a).

Interestingly, we have previously shown that expression of Ucn itself is induced in cardiac myocytes exposed to ischemia[3] and is responsible for the protective effect of the conditioned medium obtained from ischemic cardiac myocytes.[4] To test whether Ucn release was also responsible for the induction of Kir 6.1 during ischemia/reoxygenation, cardiac myocytes were exposed to ischemia/reoxygenation in the presence of an α-helical CRH peptide that blocks access of Ucn (and other CRH-related peptides) to CRH receptors. As shown in Figure 3b, addition of α-helical CRH greatly attenuated the response of the Kir 6.1 channel to ischemia/reoxygenation. When taken together with the ability of Ucn to induce Kir 6.1 expression and its release during ischemia/reoxygenation, this finding strongly suggests that the induc-

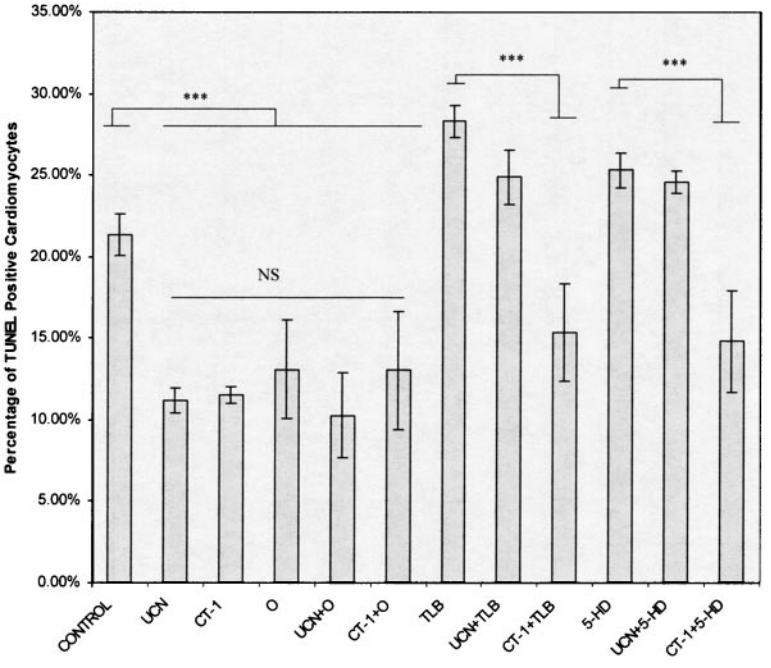

Figure 4. Cell death assays of neonatal primary cardiomyocytes exposed to simulated ischemia for 4 hours after treatment with Ucn (10^{-8} mol/L), CT-1 (10^{-6} mol/L), the potassium channel blocker tolbutamide (TLB) (10^{-7} mol/L), the potassium channel opener (O) (10^{-8} mol/L) cromakalim, or the specific mitochondrial blocker 5-HD (5×10^{-9} mol/L) for 2 hours. Values are averages of three independent experiments; bars, SD. ***$P<0.001$.

tion of Kir 6.1 during ischemia/reoxygenation[15] is mediated by Ucn.

Role of the K_{ATP} Channel in the Protective Effect of Ucn

Having established that Ucn causes a specific increase in K_{ATP} channel gene expression, we wished to determine whether such channels were of any functional significance in the protective effect of Ucn. We therefore examined the effect of a general opener of such channels, cromakalim or the K_{ATP} channel blocker tolbutamide, when added to cultured cardiac myocytes exposed to ischemia either alone or in the presence of Ucn.

In these experiments, both CT-1 and Ucn when added alone provided ≈50% protection against cell death induced by simulated ischemia in accordance with our previous results.[3,4,7,16] A similar degree of protection was also observed with the K_{ATP} channel opener cromakalim, whereas addition of tolbutamide enhanced the damaging effect of ischemia (Figure 4). Most interestingly, addition of tolbutamide prevented the protective effect of Ucn, suggesting that K_{ATP} channels are indeed involved in this protective effect. Moreover, the effect on Ucn was a specific one, given that tolbutamide had no effect on the protective effect of CT-1, which parallels the lack of effect of CT-1 on Kir 6.1 expression.

Tolbutamide is generally considered to be a sarcolemmal K_{ATP} channel blocker, although inhibition of mitochondrial K_{ATP} channels has been reported.[17] However, it is generally believed that it is the mitochondrial channel that is responsible for the cardioprotective effect of K_{ATP} channel opening.[11,18] We therefore wished to determine whether the protective effect of Ucn would be prevented by 5-hydroxydecanoate (5-HD), which is a selective mitochondrial K_{ATP} channel blocker. In these experiments, 5-HD induced enhanced cell death in cardiac myocytes exposed to simulated ischemia. Most interestingly, 5-HD treatment completely abrogated the protective effect of Ucn in exactly the same manner as tolbutamide (Figure 4). Moreover, this effect of the mitochondrial channel blocker was specific, because the protective effect of CT-1 was unaffected by 5-HD in a manner similar to the lack of effect of tolbutamide.

Effect of Inhibiting Kir 6.1 on the Response to Simulated Ischemia

The above experiments demonstrate that K_{ATP} channels are involved in the protective effect of Ucn but cannot identify the precise channel involved. However, if the induction of the Kir 6.1 subunit is involved in the Ucn protective effect, then inhibition of Kir 6.1 activity should enhance damage during simulated ischemia. As shown in Figure 5, this is indeed the case, with transfection of a dominant negative mutant of Kir 6.1[19] dramatically enhancing cell death in cardiac myocytes exposed to simulated ischemia. To our knowledge, this is the first demonstration of a protective

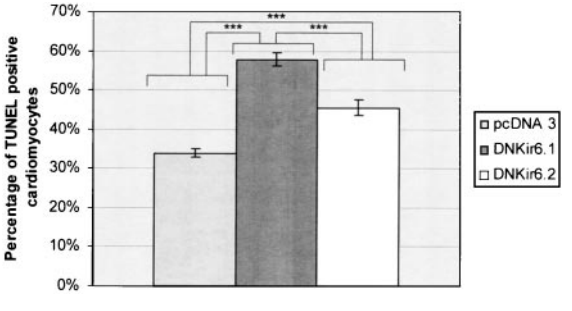

Figure 5. Cell death assays of cardiomyocytes exposed to simulated ischemia after transfection with control expression vector (pcDNA3) or the same vector containing dominant negative forms of Kir 6.1 or Kir 6.2. Values are the average of three independent experiments; bars, SD. *** $P<0.001$. TUNEL indicates terminal deoxynucleotidyltransferase–mediated dUTP nick-end labeling.

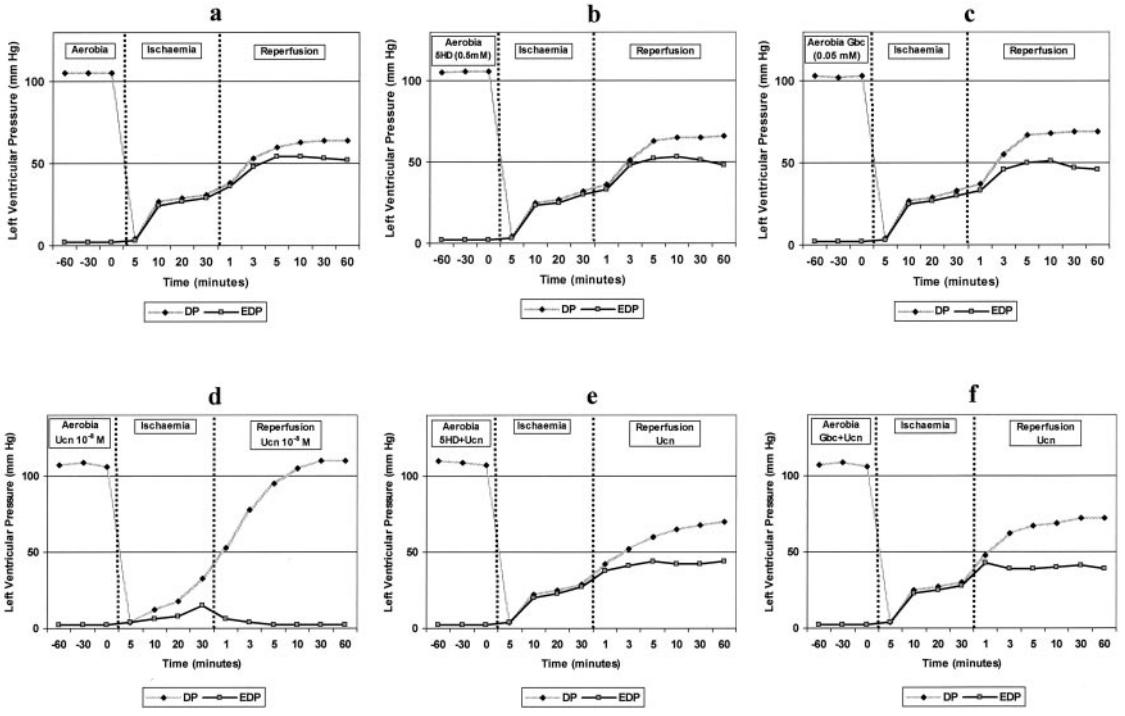

Figure 6. Effects of 5-HD, glibenclamide (Gbc), and Ucn on DP and EDP during I/R (30 minutes of global ischemia followed by 60 minutes of reperfusion) in the isolated rat heart. DP and EDP in control heart exposed to I/R are shown in panel a; changes in DP and EDP induced by 5-HD (0.5 mmol/L) and glibenclamide (0.05 mmol/L) given during the aerobic phase before I/R, and Ucn given before ischemia and during reperfusion are reported in panels b, c, and d, respectively. Hemodynamic effects of Ucn given before and after ischemia in isolated hearts pretreated with 5-HD and glibenclamide are shown in panels e and f, respectively.

role for the Kir 6.1 channel in ischemia. Moreover, the damaging effect of inhibiting Kir 6.1 was more dramatic than that observed with inhibition of Kir 6.2, which has previously been shown to have a protective effect in cells exposed to chemical hypoxia-reoxygenation.[20]

Role of the K_{ATP} Channel in the Action of Ucn in the Intact Heart

To determine whether a similar role for K_{ATP} channel in the protective effect of Ucn could be observed in the whole heart as well as in cultured cardiac cells, we used Langendorff-perfused hearts in which we have previously observed a protective effect of Ucn against the damaging effects of I/R.[5,10] In this perfused heart system, treatment with Ucn resulted in a clear increase in the proportion of cardiac myocytes staining positively with an antibody to Kir 6.1, from 1.5±0.4% in isolated hearts perfused with buffer solution only to 15.8±0.8% in hearts perfused with Ucn. Hence, Ucn induces Kir 6.1 expression in the intact heart ex vivo as well as in cultured cardiac cells.

To investigate the role of the K_{ATP} channel in the protective effect of Ucn, we used either the general K_{ATP} channel blocker glibenclamide or the specific mitochondrial blocker 5-HD in the Langendorff-perfused heart. Mechanical function of the perfused heart exposed to ischemia/reoxygenation was used as an end point, given that we have previously demonstrated a strong protective effect of Ucn in this assay.[10]

Developed pressure (DP) and end diastolic pressure (EDP) in control hearts exposed to I/R are shown in Figure 6a. In the hearts treated with Ucn before ischemia and during reperfusion (Figure 6d), Ucn significantly reduced the progressive rise of EDP observed during ischemia (13.5±3.2 mm Hg after 30 minutes of ischemia; $P<0.05$ versus I/R control) and allowed a complete recovery of EDP after reperfusion. Furthermore, Ucn produced a rapid recovery of DP, which began after only 1 minute of reperfusion (52.8±7.2 mm Hg; $P<0.01$ versus I/R control) and which progressively improved throughout reperfusion until complete normalization (110.4±12.8 mm Hg; $P<0.01$ versus I/R control).

Administration of both the mitochondrial (Figure 6b) and the general (Figure 6c) K_{ATP} blocker during aerobic perfusion had no significant effect on the hemodynamics of treated hearts exposed to I/R compared with control hearts (Figure 6a). In contrast, the pretreatment with either 5-HD or glibenclamide before Ucn infusion strongly reduced, although it did not abolish, the beneficial effects of Ucn on the functional recovery of isolated hearts observed during ischemia and reperfusion (Figure 6e and 6f; $P<0.001$ versus hearts treated with Ucn before and after ischemia).

Hence, the mitochondrial and the general K_{ATP} blockers given before arterial ligation have no effect on DP and EDP in control hearts exposed to I/R, but prevent the complete recovery of EDP and DP induced by the pre- and postischemic treatment with Ucn.

These experiments therefore demonstrate that the K_{ATP} channel is involved in the protective effect of Ucn in the intact heart. However, in view of the rapid time course of protection with Ucn in the intact heart, we wished to investigate whether de novo protein synthesis is required for the protective effect of Ucn in the intact heart as occurs in

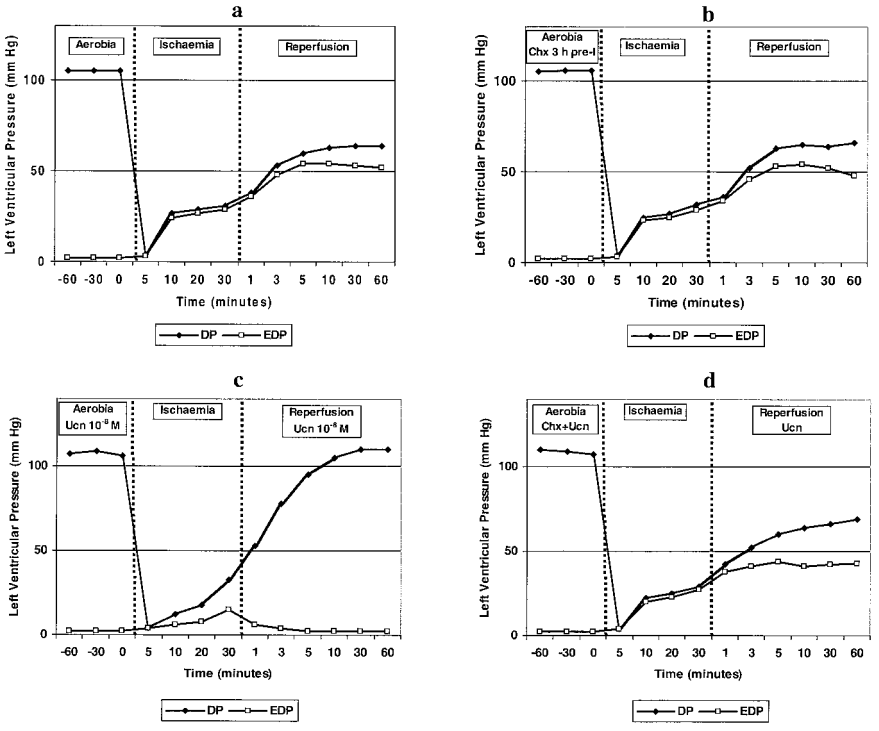

Figure 7. Effect of cycloheximide (1.0 mg/kg) on the hemodynamic effects of Ucn in the intact heart. a, Control I/R. b, Pretreatment with cycloheximide (Chx) for 3 hours before heart isolation. c, Infusion of Ucn, pre- and postischemia. d, Pretreatment with cycloheximide before heart isolation and infusion of Ucn, pre- and postischemia.

vitro.[8] We therefore tested the effect of the protein synthesis inhibitor cycloheximide on the hemodynamic recovery induced by Ucn. As illustrated in Figure 7, cycloheximide completely blocked the protective effect of Ucn ($P<0.001$) while having no effect when added alone. Hence, de novo protein synthesis is indeed required for the protective effect of Ucn in the intact heart, as in cultured cells.

Discussion

In this report we have demonstrated, for the first time, that Ucn can modulate the expression of specific genes in cardiac myocytes and that, in particular, it induces enhanced expression of the inward rectifier potassium channel Kir 6.1 subunit in both cultured cardiac cells and in the intact perfused heart. Moreover, we have shown that a generalized blocker of potassium channel opening and a blocker that specifically blocks the mitochondrial potassium channel both inhibit the cardioprotective effect of Ucn.

A number of reports have implicated the ATP-sensitive potassium channel in cardiac protection against ischemia and, in particular, in the phenomenon of preconditioning in which a series of short ischemic episodes provides protection against a subsequent more prolonged ischemia.[9,11,12] Thus, compounds that induce opening of the K_{ATP} channel result in cardiac protection mimicking preconditioning, whereas compounds that block the channel prevent the protective effect of preconditioning. On the basis of the use of inhibitors believed to be specific for sarcolemmal or mitochondrial K_{ATP} channels, it is generally believed that it is the mitochondrial K_{ATP} channel that is involved in the protective effect.[9,18] The molecular nature of this channel is at present unclear. Thus, it has been shown by using immunoblot analysis of subcellular fractions and electron microscopy that Kir 6.1 is present predominantly on the inner mitochondrial membrane,[21] which suggests that Kir 6.1 or a closely related protein is part of the mitochondrial channel. However, another study did not observe this localization and showed no suppression of mitochondrial K_{ATP} channel activity in cardiocytes infected with viruses expressing dominant negative forms of Kir 6.1 or Kir 6.2,[22] whereas another recent study did not detect Kir 6.1 in either the sarcolemmal or the mitochondrial fraction.[23] Therefore, it may be that the mitochondrial channel contains a novel potassium channel protein that has not yet been cloned.

This controversy illustrates the fact that the role of the potassium channel in cardiac protection and preconditioning has been entirely based on the effects of pharmacological compounds that are believed to induce channel opening or closing. In contrast, we present here the first evidence that a cardioprotective agent, Ucn, is able to specifically induce enhanced expression of the Kir 6.1 potassium channel subunit and that its protective effect is inhibited by generalized and mitochondrial-specific blockers of this channel, which suggests that functional potassium channels are involved in the protective effect of Ucn.

Interestingly, the expression of Kir 6.1 increased on treatment with Ucn both in vitro and in the intact heart. Moreover, inhibition of Kir 6.1 with a dominant negative mutant confirmed that it has a protective effect during ischemia; to our knowledge, this is the first time this effect has been demonstrated.

We also demonstrate that the protective effect of Ucn requires de novo protein synthesis in the intact heart, as observed in vitro.[8] Hence, the enhanced synthesis of Kir 6.1 and perhaps other proteins is required for the protective effect of Ucn. In this regard, it is of interest that we observed enhanced synthesis of PKCϵ in the gene chip screen (Table). As PKCϵ has been shown to have a protective effect in

cardiac ischemia,[24] its induction may contribute to the protective effect of Ucn. Moreover, because PKC has been shown to stimulate the activity of K_{ATP} channels,[25,26] it may also act in this way to enhance the effect of increased K_{ATP} channel synthesis. Nonetheless, our results with the K_{ATP} channel blockers and with cycloheximide indicate that the protective effect of Ucn is clearly dependent both on the K_{ATP} channel and on de novo protein synthesis.

In our experiments, both Kir 6.2 and the SUR 1 and 2 subunits show no change in expression with Ucn. Although this confirms the specificity of the change in expression of Kir 6.1 that we have observed, it raises the question of how such increased expression might result in enhanced levels of functional potassium channels given that Kir 6.X proteins need to associate with SUR proteins in a 1:1 stoichiometry. This may reflect an excess of SUR proteins in the cell, which allows more functional receptors to form when expression of Kir 6.1 is specifically enhanced. Similarly, in view of the controversy over the nature of the mitochondrial K_{ATP} channel, it is unclear whether enhanced expression of Kir 6.1 would specifically result in increased levels of mitochondrial channels or whether the expression of a yet-unidentified specific component of the mitochondrial channel would be required. In this regard, it is of interest that Ucn has previously been shown to activate calcium-sensitive potassium channels in smooth muscle cells,[27] indicating that its modulation of potassium channels is not confined to a single channel or cell type.

Enhanced expression of Kir 6.1 without any corresponding change in the expression of Kir 6.2 or of SUR was also observed in myocardial ischemia.[15] This is of interest because it indicates that expression of Kir 6.1 can be dissociated from that of the related Kir 6.2 or of SUR in several different treatments. Moreover, we have previously demonstrated that the expression of Ucn is induced in cardiac myocytes exposed to ischemia[3] and that it is released into the medium and is responsible for the protective effect of conditioned medium derived from cardiac myocytes exposed to ischemia.[4] In the experiments described here, we have shown for the first time that expression of Kir 6.1 is also induced by exposure of cultured cardiac cells to ischemia/reoxygenation. Moreover, addition of α-helical CRH, which would block the action of Ucn, also blocks the induction of Kir 6.1 by ischemia/reoxygenation. Therefore, this suggests that Ucn released in cardiac ischemia may be responsible for the specific induction of Kir 6.1 that occurs both in cultured cardiac cells and in the intact heart exposed to ischemia.[15]

Whatever the case, our studies have demonstrated for the first time that a known cardioprotective agent can increase K_{ATP} channel gene expression and that the protective effect of Ucn requires K_{ATP} channel opening.

Acknowledgments

This work was supported by the British Heart Foundation. A.S. is a British Heart Foundation Intermediate Fellow, A.C. is supported by a British Heart Foundation PhD Studentship. We thank Drs Iain Greenwood (tolbutamide and cromakalim) and Rao Sivaprasadaro (anti-SUR 1 antibody).

References

1. Donaldson C, Sutton S, Perrin MH, et al. Cloning and characterisation of human urocortin. *Endocrinology*. 1996;137:2167–2170.
2. Vaughan J, Donaldson C, Bittencourt J, et al. Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature*. 1995;378:287–292.
3. Okosi A, Brar BK, Chan M, et al. Expression and protective effects of urocortin in cardiac myocytes. *Neuropeptides*. 1998;32:167–171.
4. Brar BK, Stephanou A, Okosi A, et al. CRH-like peptides protect cardiac myocytes from lethal ischaemic injury. *Mol Cell Endocrinol*. 1999;158:55–63.
5. Brar BK, Jonassen AK, Stephanou AS, et al. Urocortin protects against ischemic and reperfusion injury via a MAPK-dependent pathway. *J Biol Chem*. 2000;275:8508–8514.
6. Sheng Z, Knowlton K, Chen J, et al. Cardiotrophin 1 (CT-1) inhibition of cardiac myocyte apoptosis via a mitogen activated protein kinase dependent pathway. *J Biol Chem*. 1997;272:5783–5791.
7. Brar BK, Stephanou A, Liao Z, et al. Cardiotrophin-1 can protect cardiac myocytes from injury when added both prior to simulated ischaemia and at reoxygenation. *Cardiovasc Res*. 2001;51:265–274.
8. Brar BK, Railson J, Stephanou A, et al. Urocortin increases the expression of heat shock protein 90 in rat cardiac myocytes in a MEK 1/2-dependent manner. *J Endocrinol*. 2002;172:283–293.
9. Scarabelli TM, Pasini E, Stephanou A, et al. Urocortin promotes hemodynamic and bioenergetic recovery and improves cell survival in the isolated rat heart exposed to ischemia/reperfusion. *J Am Coll Cardiol*. 2002;40:155–161.
10. O'Rourke B. Myocardial K_{ATP} channels in preconditioning. *Circ Res*. 2000;87:845–855.
11. Takano H, Tang X-L, Bolli R. Differential role of K_{ATP} channels in late preconditioning against myocardial stunning and infarction in rabbits. *Am J Physiol Heart Circ Physiol*. 2000;279:H2350–H2359.
12. Takashi E, Wang Y, Ashraf M. Activation of mitochondrial K_{ATP} channel elicits late preconditioning against myocardial infarction via protein kinase C signalling pathway. *Circ Res*. 1999;85:1146–1153.
13. Bernardo NL, Okubo S, Maaieh MM, et al. Delayed preconditioning with adenosine is mediated by opening of ATP-sensitive K^+ channels in rabbit heart. *Am J Physiol*. 1999;277:H128–H135.
14. Ndisdang D, Morris PJ, Chapman C, et al. The HPV activating transcription factor Brn-3a is overexpressed in CIN3 cervical lesions. *J Clin Invest*. 1998;101:1687–1692.
15. Akao M, Otani H, Horie M, et al. Myocardial ischemia induces differential regulation of K_{ATP} channel gene expression in rat hearts. *J Clin Invest*. 1997;100:3053–3059.
16. Stephanou A, Brar B, Heads R, et al. Cardiotrophin-1 induces heat shock protein accumulation in cultured cardiac cells and protects them from stressful stimuli. *J Mol Cell Cardiol*. 1998;30:849–855.
17. Smith PA, Proks P, Moorhouse A. Direct effects of tolbutamide on mitochondrial function, intracellular Ca^{2+} and exocytosis in pancreatic β-cells. *Eur J Physiol*. 1999;437:577–588.
18. Gross GJ, Fryer RM. Sarcolemmal versus mitochondrial ATP-sensitive K^+ channels and myocardial preconditioning. *Circ Res*. 1999;84:973–979.
19. Cui Y, Giblin JP, Clapp LH, et al. A mechanism for ATP sensitive potassium channel diversity: functional coassembly of two pore-forming subunits. *Proc Natl Acad Sci U S A*. 2001;98:729–734.
20. Jovanovic A, Jovanovic S, Lorenz E, et al. Recombinant cardiac ATP-sensitive K^+ channel subunits confer resistance to chemical hypoxia-reoxygenation injury. *Circulation*. 1998;98:1548–1555.
21. Suzuki M, Kotake K, Fujikura K, et al. Kir 6.1 a possible subunit of ATP sensitive K^+ channels. *Biochem Biophys Res Commun*. 1997;241:693–697.
22. Seharaseyon J, Ohler A, Sasaki N, et al. Molecular composition of mitochondrial ATP-sensitive potassium channels probed by viral Kir gene transfer. *J Mol Cell Cardiol*. 2000;32:1923–1930.
23. Miki T, Suzuki M, Shibasaki T, et al. Mouse model of Prinzmetal angina by disruption of the inward rectifier Kir 6.1. *Nat Med*. 2002;8:466–472.
24. Mackay K, Mochly-Rosen D. Localization, anchoring, and functions of protein kinase C isozymes in the heart. *J Mol Cell Cardiol*. 2001;33:1301–1307.
25. Ito K-i, Sato T, Arita M. Protein kinase C isoform-dependent modulation of ATP-sensitive K^+ channels during reoxygenation in guinea-pig ventricular myocytes. *J Physiol*. 2001;532:165–174.
26. Korge P, Honda HM, Weiss JN. Protection of cardiac mitochondria by diazoxide and protein kinase C: implications for ischemic preconditioning. *Proc Natl Acad Sci U S A*. 2002;99:3312–3317.
27. Petkova-Kirova PS, Gagov HS, Duridanova DB. Urocortin hyperpolarizes stomach smooth muscle via activation of Ca^{2+}-sensitive K^+ currents. *J Muscle Res Cell Motil*. 2000;21:639–645.