The Urocortins: Mechanisms of Cardioprotection and Therapeutic Potential

K.M. Lawrence* and D.S. Latchman

Medical Molecular Biology Unit, Institute of Child Health, University College, London, UK

Abstract: The study of the Urocortin family of peptides is becoming increasingly important clinically, as new discoveries have revealed that their roles in the body are extremely diverse. They range from being involved in the aetiology of affective disorders, boosting the immune system, to cardioprotection during ischaemia and reperfusion injury. Therefore, it is important to understand how these peptides become activated, how they activate different cell types and finally their intracellular signalling pathways. Such studies may enable scientists to develop clinical therapies based on their mechanism of action, which may be used to alleviate a diverse range of pathologies.

Key Words: Corticotrophin-releasing hormone, Urocortin, cardiac ischaemia and reperfusion injury, Affymetrix gene chip microarrays, affective disorders, cardiomyocyte mitochondria.

INTRODUCTION

The discovery and subsequent understanding of an endogenous pathway which the body uses to alleviate a particular insult, has been widely exploited in medicine to produce potent therapies which can mimic or modulate these responses. This statement is no where more true than in the heart, where the discovery of the phenomenon of ischaemic preconditioning, where multiple brief mild ischeamic insults were demonstrated to protect the heart from a much more severe ischeamia [1], led to an explosion in the discovery and subsequent study of endogenous cardioprotective agents.

An important emerging family, which falls into this category are the corticotrophin releasing hormone (CRH) related peptides, the Urocortins (Ucn). The prototype peptide CRH is defined as a hypothalamic activator of the pituitary-adrenal axis during stress [2,3]. CRH was first isolated from bovine hypothalamus almost 25 years ago [4]. Although the stress response in mammals is finely tuned and exquisitely complex, molecules involved in its pathway can be traced back through evolutionary time. For example, there are fish and amphibian homologues of CRH, urotensin 1 and sauvagine, respectively [5].

Recently, a new member of this family, Ucn I, was cloned from rat midbrain [6-8]. It was so called because of its high sequence homology to both urotensin 1 and CRH, with which it shares 65% and 45% homology at the amino acid level, respectively (Fig. **1**). This family is presently expanding further and two new Ucns have been identified from mouse genomic libraries; Ucn II and Ucn III [9,10] and equivalent sequences have been identified from human libraries and genome databases; Stresscopin Related Peptide (SRP) and Stresscopin (SCP), which are most closely related to Ucn II and Ucn III respectively [11,12,]. Like CRH, the small active forms of the Ucns are derived from a much larger prohormone, by a process of selective enzymatic cleavage [8]. Active peptide can then produce a response on its target tissue.

These peptides are widely distributed both in the central nervous system and peripheral organs. Throughout the brain, high densities of CRH immunoreactive neurones have been found in the paraventricular nucleus of the hypothalamus. Extra hypothalamic neurones containing CRH have also been found in amygdala, hippocampus and cerebellum [13-17]. In brain, CRH coordinates behavioural responses to food intake and may impair learning and memory. It is also thought to be psychotropic, being both anxiogenic and a depressant and may be involved in the genesis of anorexia and bulimia [18-20].

Ucn I however, displays a different pattern of distribution. High mRNA levels have been found in Edinger-Westphal nucleus with lesser amounts in the hippocampus and basal ganglia including the substantia nigra [21,22]. Ucn II distribution is restricted to the supraoptic and arcuate nuclei of the hypothalamus, locus coeruleus, brain stem and spinal cord [9,12]. In contrast Ucn III is expressed in the bed nucleus of the stria terminalis, the medial nucleus of the amygdale, the hypothalamus and brain stem [23].

Peripherally, CRH and the Ucns are thought to be able to influence immune functions. *In vitro* CRH has been demonstrated to stimulate both B and T-lymphocyte proliferation [24]. Exogenously applied CRH can reduce inflammation. Although the role of endogenous Ucn I is not yet clear, it can at least mimic some of the actions of CRH on the immune system [25], as administration of Ucn I can also suppress inflammation [26].

All the Ucns are highly abundant in, and even exhibit heterogeneous distribution amongst the four chambers of the heart [27]. Interestingly however, several research groups have been unable to detect CRH in heart. A number of pieces of evidence led to the proposal that in heart, Ucn I may be a potent cardioprotective agent during Ischaemia and reperfusion injury (I/R). For example during simulated I/R in primary cardiomyocytes the levels of Ucn I mRNA increased

*Address correspondence to this author at the Medical Molecular Biology Unit, Institute of Child Health, University College, London, UK;
E-mail: k.lawrence@ich.ucl.ac.uk

1389-5575/06 $50.00+.00

Fig. (1). Amino acid alignment of rodent CRH related peptides. Areas of high sequence homology have been highlighted.

dramatically, based on a sensitive 3'RACE assay [28]. Furthermore, this increase was also seen at the protein level as demonstrated by Western blot studies [29], implying both an increase In Ucn I mRNA and an increase in cleavage to the active form. Indeed conditioned media derived from cardiomyocytes exposed to I/R, were able to protect naïve cells from the damaging effects of I/R. Furthermore, this effect could be blocked by Ucn antagonists [28,29]. These findings led to the suggestion that endogenous Ucn I could be up regulated during I/R and released into the local environment where it could bind back onto the cardiac sarcolemma, producing an effect in an autocrine /paracrine manner [8,30].

To date, most work has been performed on Ucn I in relation to cardioprotection against I/R injury. For example, Ucn I exogenously applied to primary cardiomyocytes during simulated I/R, in the Langendorff perfused ex vivo heart model and in whole heart *in vivo*, is protective when given during ischaemia. Of more clinical relevance however, it is also highly protective when given at reperfusion after the ischaemic episode [31-33]. This is important as it has been demonstrated in many species, including humans, that most damage to cardiac tissue occurs at this point. These findings have recently been confirmed *in vitro* and in the Langendorff perfused *ex vivo* heart for Ucn II and Ucn III also [34,35].

Clinically it is important to look at each point along the pathway of Ucns cardioprotective mechanism of action, in order to look for targets where new therapies may be developed. The goal of this would be to mimic, or enhance further the Ucns cardioprotective action. Thus we will start at the point where active Ucns are first generated.

Prohormones

The generation of an active peptide or hormone, from its inactive longer form, represents the first point at which the effects of CRH and the Ucns may be manipulated clinically. By regulating the concentration of their active forms, for example reducing the amount of active CRH in certain brain regions, may represent a therapy for affective disorders. In contrast in the heart, elevating active Ucn I, II and III so that they may be released and bind back onto the cardiomyocyte sarcolemma in an autocrine /paracrine manner would have potent cardioprotective properties against I/R injury. To date however, little is known about the processing of CRH or the Ucns in brain and virtually nothing of this process in the heart.

There is a growing family of enzymes, the prohormone convertases (PCs) which are responsible for the cleavage of large prohormones into smaller active forms [36]. It is known that the active form of CRH, the 41 amino acid-containing peptide is cleaved from the 186 amino acid, prohormone by this family of enzymes, but very little is known about processing of the Ucns. Thus, once identified, it may be possible to synthesise selective activators or inhibitors of these enzymes to produce the desired elevation or decrease in a particular active form. Of course one confounding factor is the strong possibility that these enzymes may not be specific for the CRH family but have multiple prohormone targets. Thus, there is a large question mark over the selectivity of this approach. For example the isozyme PC2 is hypothesised to convert pro CRH into active peptide as well as multiple pro-neuropeptides into active peptides that function as neurotransmitters. However, in PC2 deficient mice, the processing of pro CRH in brain and the periphery was not affected suggesting that this isozyme is not solely responsible for the processing of CRH [37]. Thus the approach of selectively inhibiting or increasing the amount of active peptide species from inactive forms is still very much in its infancy.

CRH Receptors

An obvious target clinically in which to enhance or antagonise an effect seen by an endogenous ligand would be at its specific high affinity receptor.

The first CRH receptor to be cloned was a 415 amino acid seven transmembrane domain G-protein coupled receptor [38,39]. From this original CRHR1 receptor sequence, hybridisation probes were used under low to moderate stringency to clone a second CRHR2 receptor which was derived from a separate gene [40]. Subsequent binding assays and structure affinity relationships using chimeric peptides have been used to investigate receptor subtype selectivity. These studies revealed that the N-terminal region of both the CRHR1 and CRHR2 is responsible for ligand binding [41]. Although CRH and Ucn I bind to both CRHR1 and CRHR2, Ucn I displayed more than 100 fold higher binding affinity for the CRHR2 than did CRH [7]. In contrast Ucn II and Ucn III, bind exclusively to the CRHR2 subtype [9,12] (Fig. **2**).

Further variety in receptor structure is achieved by extensive alternative RNA splicing [42]. The CRHR1 gene is expressed as eight subtypes 1a-h. The CRHR2 gene has only three isoforms α,β,γ, which are species specific. All CRH receptor isoforms to date, conform to the classic G protein coupled, seven hydrophobic transmembrane spanning domain structure. Upon activation, they produce an increase in cAMP and are linked to the cholera toxin sensitive stimulatory type of G protein [44,45]. This suggests that once the ligand has bound to its receptor, downstream signalling should follow a common pathway. However, some interesting studies have found that different G protein species are coupled to different receptor subtypes, presumably eliciting different downstream responses, thus increasing further the variety and complexity of the responses and signalling produced by the different CRH family members.

Ucn I has several effects on heart and cardiovascular physiology due to its action on CRHR2, the only receptor subtype found in cardiac tissue. These include hypotension, increased cardiac contractility, and stroke volume output

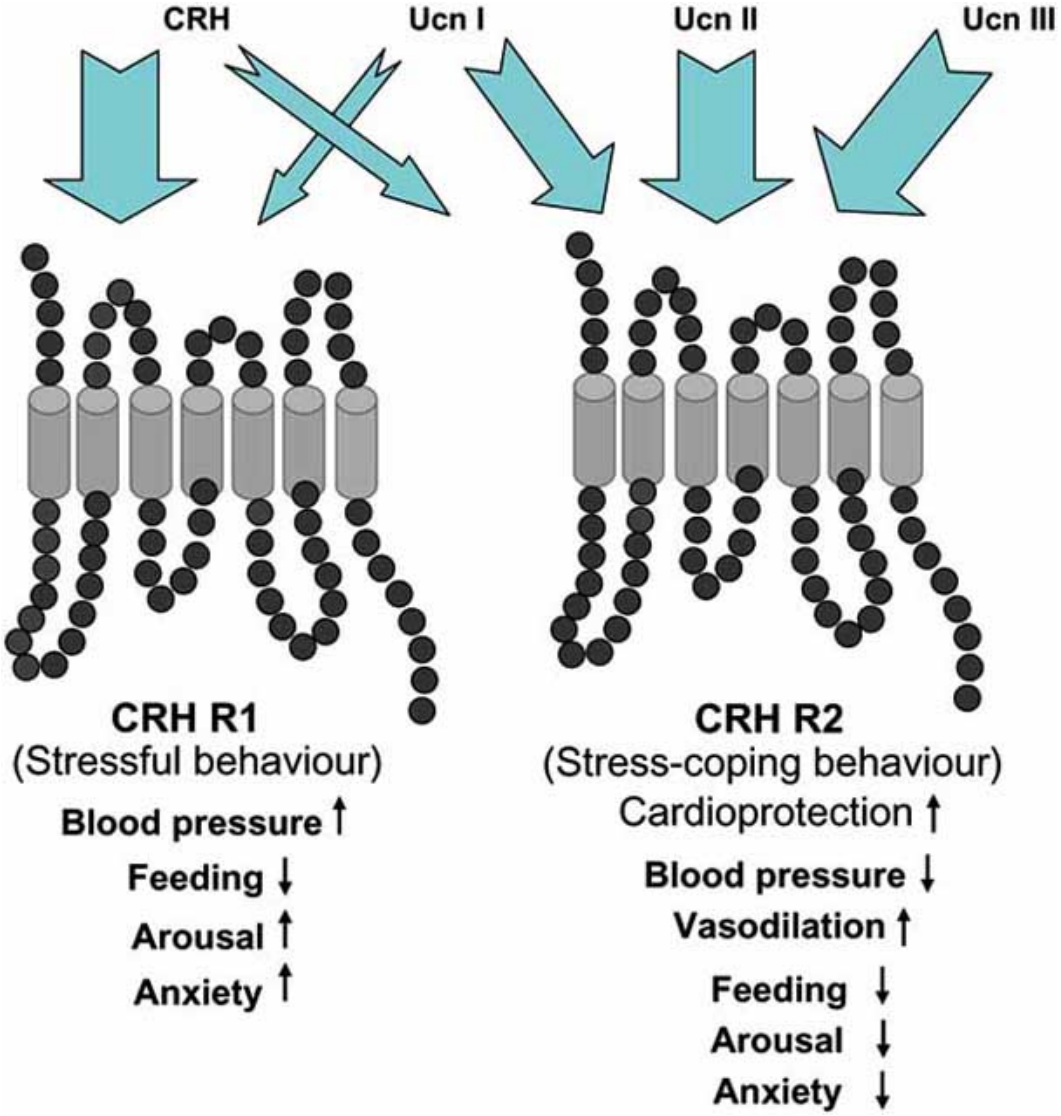

Fig. (2). Effects of stimulation of the two CRH receptor subtypes by CRH family members. The thickness of the arrows indicates binding affinity for the different CRH peptides at the CRH receptors. Thus, CRH binds with a higher affinity to CRHR1 than CRHR2. Ucn I however, binds with a higher affinity to CRHR2 than CRHR1. Ucn II and Ucn III bind exclusively and with high affinity to CRHR2.

[27]. A positive inotropic action and a vasodilator action of the Ucns may be beneficial for the improvement of vascular spasm or stenosis also (Fig. **2**).

Clinically it would be very useful to selectively activate or block CRH receptor subtypes in a tissue specific manner. For example, in brain, selectively antagonising the effects of CRH in producing depression and anxiety, *via* CRHR1, may be useful novel therapies for these affective disorders. In contrast as in heart, selective agonists for the CRHR2 receptor would be preferred as this would enhance cardioprotection against I/R injury. The drugs could be used as a prophylactic in patients with a high risk of cardiac arrest, or given at reperfusion after an attack has happened.

Antagonists to the CRH receptors, such as alpha helical CRH and astressin, have been demonstrated to be potent but of limited use as they are non-selective, binding to both CRHR1 and CRHR2 with equal affinity. However, some selective antagonists of CRHR1 such as R278995 and CRA0405 have now been developed. These have potent anxiolytic and antidepressant like activities. Recent advances have also been seen with analogues to amphibian sauvagine being used in the development of CRH receptor subtype specific agonists. Again, looking at structure affinity relationships, altering amino acid residues 11, 12 and 13 of synthetic sauvagine improved CRH2R selectivity, although not to the level of CRHR2 selective peptides such as Ucn II and Ucn III [46]. Further selectivity for the CRHR2 receptor using sauvagine analogues was achieved by modifications of residues 35 and 39. Sites responsible for CRHR2 selectivity were finally pinpointed to residue 35 along with 11, 12, and 13 in sauvagine [46]. Importantly clinically, these analogues have higher efficacies than the parent compound [46]. Thus some progress has been made in the development of synthetic peptides which may be more selective and efficacious than their parent compounds.

Kinase Pathways Involved in the Cardioprotective Effects of Urocortin

The consequences of the binding of Ucn I and its homologues to cardiomyocytes are complex and can be divided into rapid effects and more longer term altered gene expression effects, all of which eventually lead to cardioprotection from I/R injury. The ability of Ucn I and its homologues to protect the heart from I/R injury is now overwhelming. However, the precise mechanism of action of these cardioprotective agents is less well understood.

It is now known that several major kinase pathways are affected by Ucn I treatment in heart. A number of early studies using primary cardiomyocyte preparations implicated the mitogen-activated protein kinase (MAPK) as being one cardioprotective pathway employed by Ucn I [30,31,33]. A subfamily of MAPK the p42/p44 MAPK, are phosphorylated and activated by the MAPK kinase kinase (MEK 1/2) following Ucn I treatment. Interestingly, specific pharmacological inhibition of MEK 1/2 by PD 98059 abolished cardioprotection produced by Ucn I when assayed using cell death/apoptosis assays such as trypan blue exclusion, Annexin V and TUNEL positivity [30,31,33].

This abolition of cardioprotection by Ucn I, was seen when PD 98059 was given in the presence of Ucn I during simulated ischaemia but also removed Ucn's protective effect during reoxygenation [31]. Although studies using primary cardiomyocyte preparations are important, it is however, crucial to extend these investigations to the whole heart. Again the inhibition of MEK 1/2 pathway by PD 98059 blunted the ability of Ucn I to reduce infarct size during I/R in an *ex vivo* heart model employing the Langendorff perfusion apparatus and *in vivo* [33]. Recently, these findings were also seen for Ucn II and Ucn III, both *in vitro* and using the Langendorf perfused *ex vivo* heart model [34,35].

In addition to the p42/44MAPK pathway, activation of the phosphatidyl inositol 3-OH kinase (PI3K) and the serine threonine kinase Akt, its downstream effector, has also been demonstrated to preserve cardiac function and to be involved in cardioprotection produced by Ucn I during I/R. Thus chemical inhibitors of the PI3K pathway such as Wortmannin and LY 294002 have been demonstrated to block Ucn I mediated cardioprotection in both neonatal and adult cardiomyocytes [32]. Again, both Ucn I homologues also seem to work through the PI3K pathway [34,35].

Activation of both the MEK1/2 and PI3K pathways is thought to be cardioprotective because the kinases phosphorylate pro apoptotic molecules such as BCL_2-antagonist of cell death (BAD) causing it to bind 14-3-3 protein, which sequesters it from its mitochondrial target thus preventing apoptosis [47]. Furthermore, these kinase pathways inhibit the conformational change in the pro-apoptotic molecule BCL_2-associated X protein (BAX), which is necessary for its translocation to the mitochondria where it is thought to produce pores resulting in the release of cytochrome C [48, 49]. They are also thought to be responsible for preventing procaspases such as 9 and 3 from being cleaved into their active forms [50,51].

In contrast with the majority of Ucn I-mediated beneficial effects on the cardiovascular system, it also has a less desirable hypertrophic effect on the heart [52,53]. This causes cardiomyocytes to increase in size with a concomitant increase in their protein to DNA ratio with an induction in both atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). This reversion of some cells back to their embryonic state, causes the heart to beat asynchronously and in severe cases to fail altogether. However, this hypertrophy is not caused by activation of the p42/44 kinase pathway, which is crucial for cardioprotection. A recent study has found that activation of the PI3 AKt is necessary for cardiac hypertrophy produced by Ucn I [53]. Thus analogues of the Ucns may be designed to produce selective activation of the protective pathway without any concurrent hypertrophic response. However, to date, no selective activator of the individual kinase pathways exists.

A third kinase, PKC has for some time been implicated in cardioprotection during I/R injury. However its involvement is complicated by the revelation that to date, there are twelve different isozymes of PKC. These are contained within three different families: classical, atypical and novel PKCs , each phosphorylating diverse effectors and having a wide

range of tissue and subcellur distribution [54-56]. Until recently, it has been impossible to dissect the importance of individual isozymes in terms of a physiological function, as pharmacological agents lack the selectivity needed. Recently however, small peptides of 6-8 amino acids have been used to inhibit specific isozymes of PKC from binding to their specific receptor for activated C kinase (RACK) [57]. These assays take the form of inhibition of a specific isozyme of PCK translocating from a cytosolic to a membrane fraction [58,59] Pseudo RACK peptides have also been used to enhance the function of specific PKCs [59]. These data, along with studies using knock out mice and mice over expressing individual PKC isozymes has, in cardiac cells and the whole heart, strongly implicated the PKCε isozyme as the major PKC involved in cardioprotection during I/R [60-62] and in producing the phenomenon of ischaemic preconditioning [63-65]. Indeed pseudo RACK designed specifically to PKCε has been used successful as a cardioprotective agent [59].

Very recently, Ucn has been demonstrated to be a selective activator of PKCε. Ucn caused a specific translocation (which is an indicator of increased activation) of PKCε *in vitro* and in the Langendorff perfused *ex vivo* heart [66]. Furthermore, a PKCε specific inhibitor peptide, when introduced into cardiomyocytes, prior to simulated ischemia caused Ucn I to lose its cardioprotective effect [66]. This loss of cardioprotection by Ucn I was also seen in whole hearts from PKCε knockout mice when they were exposed to a 45 minute ischaemia and 30 minute reperfusion protocol. These findings indicate that the cardioprotective effect of Ucn I also involves the PKCε signalling pathway. Thus selective enhancement of this in the heart, may be an important cardioprotective pathway open to medical exploitation.

As well as effects on diverse kinase pathways, recently urocortin has also been shown to modulate L-type calcium channels [67]. Using whole- cell patch-clamp recording on isolated adult rat cardiomyocytes, Ucn I produced a concentration dependent decrease in the inward calcium current after ten minutes, which correlated with increased cell survival [67]. Unfortunately, it is unclear whether Ucn I had a direct effect on the channel moiety, or its modulation involved activation of the cardiac CRHR2 receptor, as the effect of receptor antagonists to Ucn I were not investigated in this study. These channels may represent a novel site which may be modulated to produce a cardioprotective effect during I/R injury, since there are already available numerous openers and blockers of this type of channel [67].

Altered Gene Expression and Cardioprotection

As well as acute effects on diverse kinase pathways, a part of cardioprotection by Ucn I requires de-novo protein synthesis, as protection is lost in the presence of protein synthesis inhibitors [28]. Using a candidate gene approach, expression of the cardioprotective heat shock protein 90 (hsp 90) has been shown to be induced by Ucn I [68].This effect is blocked by the MEK1/2 inhibitor PD 98059 [68]. This same approach was also used to show that Ucn I causes an increase in the expression of the cardioprotective agent Cardiotrophin -1 (CT-1) [69].

Because Ucn I was shown to alter gene expression, but also because of the obvious limitations of a study employing a candidate gene approach, the effects of Ucn I on global gene expression was investigated. The use of Affymetrix gene chip technology was used in unravelling the gene expression profile of Ucn I [70] and expression of several genes was shown to be altererd by Ucn I treatment of cardiac cells [70]. They included genes that were found to be both up-regulated and attenuated by the peptide [70]. Three gene products in particular were altered by Ucn I. Although they were seemingly unrelated in terms of functional protein produced, all three were found to be intimately involved in cardioprotection.

The first protein studied was an ATP sensitive potassium channel (Katp channel). These channels are dependent upon the cellular concentration of ATP for activation. When the concentration of ATP falls the channels open, but under normal physiological concentrations of ATP they remain closed. Thus they are sensors of the metabolic state of a cell. These channels, when open, during stressful stimuli including I/R are cardioprotective [71-77].

There are two subtypes of this channel known, each a product of a separate gene, kir6.1 and kir6.2. These are small two transmembrane spanning domain proteins which represent the pore of the channel moiety. To make the channel functional, requires another subunit derived from a totally different set of genes, the sulphonylurea receptors (SUR), so called because of their binding site for the sulphonylurea class of drugs used in the treatment of diabetes. These receptors are large, twelve transmembrane spanning domain proteins and are members of the ABC binding cassette superfamily. These subunits are responsible for sensing and binding ATP/ADP and ultimately gating the channel pore [78,79].

Ucn I specifically induced expression of the kir 6.1 potassium channel subunit, both in primary cardiomyocytes and whole heart. No differences were seen in the expression of kir 6.2 or the three alternatively spliced isoforms of SUR [70]. Using an antagonist to the mitochondrial Katp channel, 5-hydroxydecanoate (5-HD) and dominant negative constructs to kir6.1, it was possible to demonstrate that the cardioprotective effect of Ucn I was lost when kir 6.1 was inhibited [70]. In contrast openers of Katp channels such as cromakalim were cardioprotective during simulated I/R injury *in vitro* [70].

A second gene modulated by Ucn I, is $iPLA_2$. This gene product belongs to a super family of phosphoplipases represented by 3 classes; cytosolic PLA_2 ($cPLA_2$), secretory PLA_2 ($sPLA_2$) and calcium independent PLA_2 ($iPLA_2$) [80]. They are characterised on the basis of cellular localisation, substrate specificity, Ca^{++} dependency and type of lipid modulator [80]. $iPLA_2$ catalyses the breakdown of membrane phospholipids into arachidonic acid, which is a precursor of prostaglandins and leukotrienes and a minor metabolite lysophosphatidylcholine (LPC) [81].

It has been demonstrated that only the activity of the cardiac $iPLA_2$ class of PLA_2, is increased during I/R with a concomitant increase in the generation of LPC which is highly cardiotoxic [81]. Very interestingly, Ucn I was found to lower the expression levels of this enzyme over two fold and also lowered the generation of LPC in normoxic cardio-

myocytes and in those exposed to I/R, as did a specific pharmacological inhibitor of $iPLA_2$, bromoenol lactone (BEL) resulting in cardioprotection [82]. Recently, enantiomers of BEL have been synthesised, which can selectively block either of the two newly discovered isozymes of $iPLA_2$, the β and γ forms [83,84]. Interestingly, only the β selective enantiomer was found to be cardioprotective, revealing a new strata of specificity for $iPLA_2$ blockers, which may be exploited to produce highly selective cardioprotective agents [85,86].

The third gene product found to be altered by Ucn I from the gene chip study was PCKε. Ucn I , as well as activating this kinase as described above, also caused an increase in the mRNA and protein levels by over three fold [66], thus increasing the readily available translocatable pool during cardioprotection.

Cardioprotection by Ucn Involves Cardiomyocyte Mitochondria

It has been demonstrated that initially during I/R injury, cardiomyocyte mitochondrial function is compromised. There is a loss of membrane potential resulting in decrease in oxidative phosphorylation as well as increases in reactive oxygen species and the release of pro-apoptotic molecules including cytochrome C [87-89].

Why however, study cardiomyocyte mitochondria in relation to the cardioprotective effect of Ucn I? Very recently a link has been found between the genes regulated by Ucn I and cardiomyocyte mitochondria. The three proteins found to be important during Ucn's cardioprotective mechanism of action during I/R (as described above, kir 6.1, $iPLA_2$ and PKCε) are also localised to the mitochondria, based on a combination of pharmacology, Western blotting and immu-

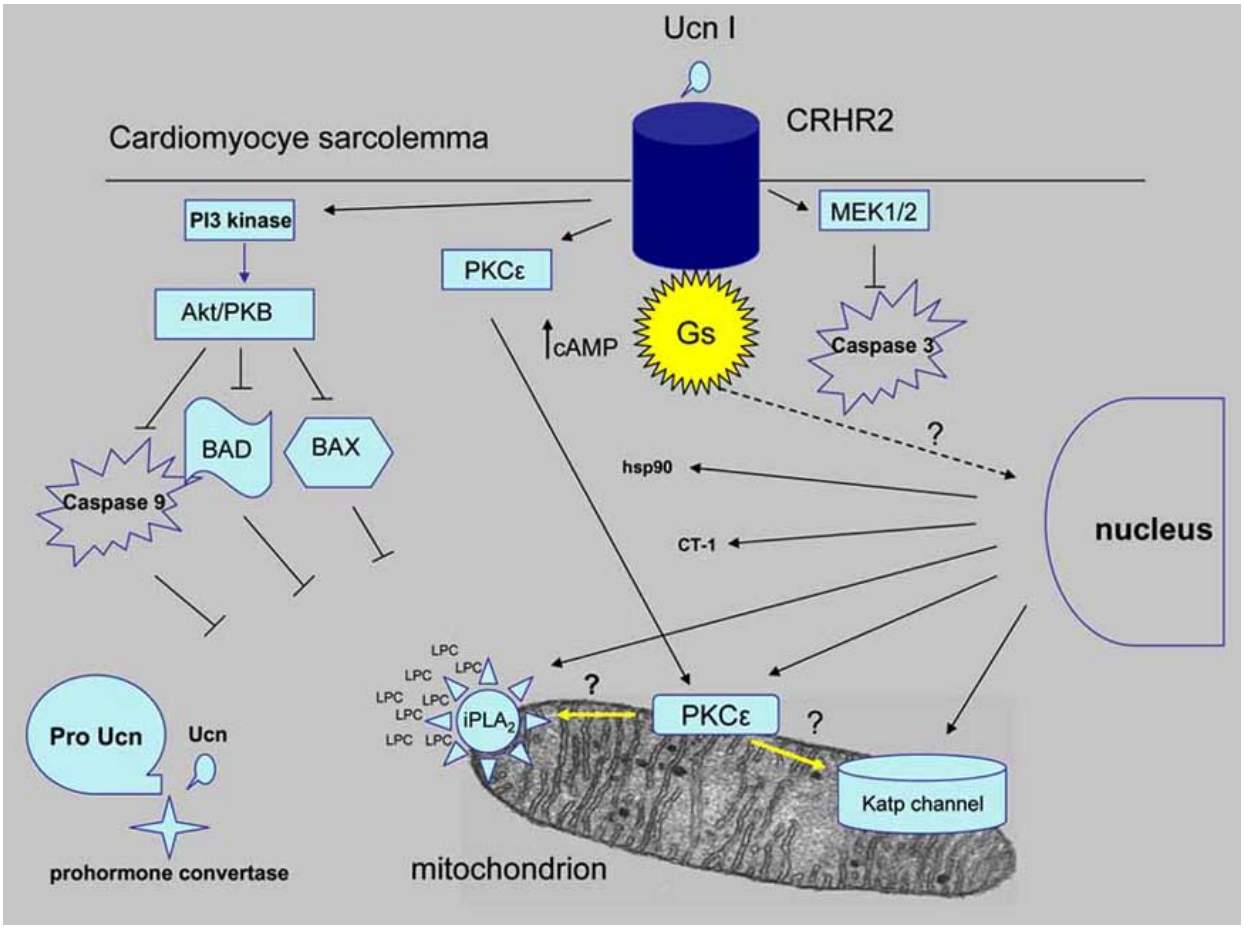

Fig. (3). Schematic diagram of the known effects of Ucn I binding to its CRHR2 receptor in cardiomyocytes. During Ischaemia and reperfusion (I/R), prohormone convertases become activated causing an increase in the concentration of cleaved active Ucn I, which is released into the surrounding milieu, where it binds back onto the sarcolemmal CRHR2 receptor. This causes activation of stimulatory G proteins (Gs) causing an activation of adenylyl cyclase and increased cyclic adenosine monophosphate (cAMP). Initially, this activates the mitogen activated kinase p42/44 (MAPK), phosphoinositde-3 kinase (PI-3K) pathways and protein kinase C epsilon (PKCε), each of which has been demonstrated to be important in early cardioprotection.

Later, unknown signals targeted to the nucleus, cause an increase in the gene expression levels of heat shock protein 90 (hsp 90), Cardiotrophin-1 (CT-1), kir6.1 and PKCε, while attenuating the gene expression of calcium insensitive phospholipase A_2 ($iPLA_2$). The latter three gene products have been demonstrated to localise to the cardiomyocyte mitochondrion where they may be protective (kir6.1, PKCε) or damaging ($iPLA_2$). To date no direct interaction between these proteins have been demonstrated at the level of the mitochondrion.

nocytochemistry [90]. Thus a clue to how Ucn I protects cardiomyocytes from I/R damage may lie at the level of the cardiomyocyte mitochondria [90].

By using the mitochondrial selective dyes (mitotracker green and TMRM) to measure damage to the mitochondrial transmembrane potential (ψm) [89], it was found that Ucn I indeed protects cardiomyocyte mitochondria from damage produced by I/R injury [90]. The protective effect on mitochondria, from I/R injury, was also observed in the presence of the Katp channel opener cromakalim and the $iPLA_2$ inhibitor BEL, suggesting that both Katp channel opening and inhibition of LPC formation, are crucial for the protection of cardiomyocyte mitochondria during I/R injury [90]. When during I/R, the mitochondrial Katp channel is blocked using 5-HD, or PKCε activation is blocked by selective inhibitor peptides, or if exogenous LPC applied to primary cardiomyocytes, mitochondrial damage is enhanced compared with I/R alone. Furthermore, crucially, the protective effect of Ucn I is also lost [90].

Interestingly, the Katp channel opener cromakalim also protects cardiomyocyte mitochondria from LPC induced damage, suggesting a possible interaction between mitochondrial Katp channels and the $iPLA_2$ metabolite LPC. As some studies suggest, this metabolite interacts with ion channels and may even be an antagonist of potassium channels [91,92]. Therefore, some protection afforded by chromakalim may be due to pharmacological competition for the same binding site as LPC. However, when 5-HD is present together with LPC, mitochondrial damage is enhanced compared to cardiomyocytes treated with either agent alone, suggesting that damage to mitochondria by LPC may also be *via* mechanisms other than Katp channels.

Thus, three end effector molecules modulated by Ucn I, are localised to cardiomyocyte mitochondria and are involved in I/R injury and cardioprotection. Furthermore, there is accumulating evidence that these three molecules can interact. For example, there is now evidence that LPC can modulate both Katp channels and PKCε and that PKCε can interact with Katp channels and $iPLA_2$ [93-98]. Significantly, PKCε has been shown to translocate to mitochondrial membranes and interact with mitochondrial proteins, including the mitochondrial permeability transition pore [99].

Although further studies are necessary to define fully the mechanism of cardioprotection produced by Ucn I, especially at the level of the mitochondrion, these organelles represent an end target where cardioprotective therapies may be developed. In fact there is an ever growing number of diseases which are thought to be related to mitochondrial damage and/or function. Much less work has been performed on the newer homologues of Ucn I, Ucn II and Ucn III in relation to their cardioprotective mechanism of action and in particular whether they are protective at the level of the mitochondrion. It will be interesting in the future to determine whether their cardioprotective pathways resemble that of Ucn I, or whether they diverge giving some novel twist to the cardioprotective story of the Ucns (Fig. **3**).

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Received: May 15, 2006 **Revised: July 04, 2006** **Accepted: July 14, 2006**