The cardioprotective effects of urocortin are mediated via activation of the SRC tyrosine kinase-STAT3 pathway

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Introduction

Ischemia/reperfusion (I/R) injury in the heart results in the necrotic and apoptotic death of irreplaceable cardiac myocytes. Apoptosis has become increasingly recognized as one of the mechanisms of cell death during I/R injury in the heart (for a review, see ref. 1), and in both human and animal studies, apoptosis has been observed in the infarcted area of the heart following acute myocardial infarction.2,3 Previously we have demonstrated that urocortin (Ucn), a small peptide belonging to the corticotrophin-releasing factor family4 can provide cardioprotection by reducing the extent of both necrotic and apoptotic myocyte cell loss caused by I/R injury.5-7

Endogenous Ucn is known to be upregulated in primary cultures of cardiac myocytes exposed to hypoxia/reoxygenation (H/R),3 as well as in the human heart undergoing cardioplogic arrest, where it protects cardiac cells via an autocrine/paracrine mechanism.5 Increased Ucn expression has also been shown in the intact rat heart exposed to ex vivo and in vivo I/R injury, where Ucn is released into the circulation by ischemic/reperfused cardiomyocytes, which are metabolically challenged, though still viable.6 Furthermore, pre-ischemic treatment with exogenous Ucn, performed both ex vivo and in vivo, reduced infarct size in the rat heart,11 with fewer apoptotic myocytes, and promoted hemodynamic and bioenergetic recovery.11 Several molecular mechanisms have been implicated in Ucn-mediated cardioprotection, including activation of mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K),12,13 down-regulation of calcium-independent phospholipase A2 (iPLA2),14 upregulation and mitochondrial relocation of protein kinase C-epsilon (PKCe),15 as well as activation of mitochondrial ATP-sensitive potassium channels (KATP).16

Src tyrosine kinase family was recently identified as a novel upstream modulator of MAP kinase subfamily, p42/p44, whose activation is required for urocortin (Ucn)-mediated cardioprotection. Src kinase was also shown to reduce apoptosis in different cancer cell lines, enhancing phosphorylation and DNA binding affinity of signal transducer and activator of transcription (STAT)3. In order to evaluate the effects of Ucn on the activation status of different STAT family members, HL-1 cardiac cells were incubated with Ucn (10 nM) for increasing periods of time. STAT3 was rapidly phosphorylated at Tyr705, while neither phosphorylation at Ser727 nor induction of total STAT3 was observed. Pretreatment with PP2, a selective inhibitor of Src tyrosine kinase, reduced the pSTAT−T705 phosphorylation and transcriptional activity induced by Ucn in a dose-dependent manner. Overexpression of STAT3 in HL-1 cardiac myocytes pretreated with Ucn reduced the magnitude of cell death as compared with Ucn treatment alone, while transfection of HL-1 cells with a STAT3 mutant functionally inactive, acting as a dominant negative (DN-STAT3), enhanced the extent of cell death in a dose-dependent manner. In line with this finding, in HL-1 cardiac myocytes overexpressing STAT3 treated with Ucn, addition of the Src kinase inhibitor PP2 reversed the cytoprotective effects of Ucn, proving that the cytoprotective effects of Ucn are also mediated via the Src-pSTAT−T705 phosphorylation pathway. By immunocytochemistry, Ucn induced nuclear translocation of pST3-T705, which was inhibited by pretreatment with PP2. Together, these data strongly suggest that Ucn can mediate cardioprotection by activating the Src-pSTAT-T705 phosphorylation pathway.

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The signal transducers and activators of transcription (STATs) are a family of transcription factors which were originally identified on the basis of their ability to transduce a signal from a cellular receptor into the nucleus and modulate the transcription of specific genes. STATs are activated by several kinases including the Janus-activated kinase (JAK) family of proteins consisting of four family members, JAK1, 2, 3, and Tyk2. STATs are also activated by growth factor receptors, particularly the epidermal growth factor receptor (EGFR), as well as by members of the Src family of kinases (SFKs), particularly c-Src. Recent studies have demonstrated that STAT1 plays a key role in promoting apoptosis in a variety of cell types, whereas STAT3 has an anti-apoptotic effect. Moreover, while STAT3 promotes cellular proliferation and is activated in a variety of tumor cells, STAT1 appears to have an anti-proliferative effect. STAT1 and STAT3 have also been shown to have opposing effects in cardiac myocytes exposed to I/R where STAT1 promotes apoptotic cell death, whereas STAT3 is cytoprotective in cardiac myocytes following I/R.

Recently, we reported that Ucn activates the Src pathway, whose activation plays a crucial role in mediating the cardioprotective effects of Ucn. Thus, the selective Src family kinase inhibitor, PP2, or a dominant negative mutant of Src, abrogated Ucn-mediated cardioprotection in cardiac cells exposed to I/R. However, an interplay between Ucn, Src, and STAT3 has not been investigated. In this report, we show that Ucn induces phosphorylation of STAT3-Tyr705 in primary cardiac myocytes, which was abolished in the presence of PP2, but not by a JAK inhibitor. Cardiac myocytes pretreated with Ucn showed enhanced nuclear relocation of pSTAT3-Tyr705, which was associated with reduced TUNEL labeling. Overexpression of a dominant negative STAT3-T705A reduced the cytoprotective effects of Ucn in primary cardiac myocytes. The inhibitory effects of STAT3-T705A on Ucn-mediated cytoprotection were reversed by overexpressing a constitutively active form of STAT3 in cardiac cells exposed to I/R. These results demonstrate for the first time that Ucn activates STAT3 via the Src pathway in cardiac myocytes and that this activation is involved in Ucn-mediated cardioprotection.

### Results

Ucn activates the Src-STAT3 pathway. HL-1 cardiac cells were incubated with Ucn (10 nM) for increasing periods of time (15, 30, and 60 min). A phospho-STAT antibody sampler kit (Phospho-Stat Pathway Sampler Kit; Cell Signaling Technology) was used in western blot analysis experiments to evaluate the effects of Ucn on the activation status of different STAT family members, including the phosphorylation of STAT1 at Tyr701 (pST1701), but not STAT1 phosphorylation at Tyr701 (pST1701). PP2 pretreatment reduced the pSTAT−T705 phosphorylation induced by Ucn in a dose-dependent manner. Ucn enhances activity of a STAT3-luciferase reporter, which was also abolished by PP2 (P2), though not by AG490 (AG).

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**Figure 1.** (A) In HL-1 cardiac cells and primary neonatal cardiac myocytes (primary) incubated with Ucn (10 nM) for increasing periods of time (15, 30, and 60 min), STAT3 phosphorylation occurred at Tyr705 (pST3705), but not STAT1 phosphorylation at Tyr701 (pST1701). (B) PP2 pretreatment reduced the pSTAT−T705 phosphorylation induced by Ucn in a dose-dependent manner. (C) Ucn enhances activity of a STAT3-luciferase reporter, which was also abolished by PP2 (P2), though not by AG490 (AG). Data represent the mean ± SE of three independent experiments. *P < 0.05.
crucial for Ucn-mediated cardioprotection. Thus, to examine whether Src tyrosine kinase activation mediates STAT3 tyrosine 705 phosphorylation (pSTAT-T705), HL-1 cardiac myocytes and neonatal ventricular cardiac myocytes were incubated with 10 nM Ucn in the presence or absence of PP2, a selective pharmacological inhibitor of Src tyrosine kinase family. Figure 1B shows that PP2 pretreatment reduced the pSTAT-T705 phosphorylation induced by Ucn in a dose-dependent manner. In contrast, the JAK inhibitor AG490 failed to reduce the Ucn-induced pSTAT-T705 phosphorylation, indicating that Ucn specifically induces pSTAT-T705 phosphorylation via a Src tyrosine kinase-dependent pathway. Next we determined whether Ucn-induced pSTAT-T705 phosphorylation is also associated with STAT3 transcriptional activity. Figure 1C shows that Ucn enhances activity of a STAT3-luciferase reporter, which was also abolished by PP2, though not by AG490.

Previous studies have shown that Ucn has cardioprotective properties in cardiac myocytes exposed to I/R injury. Therefore, we next assessed whether the cardioprotective effects of Ucn are mediated, at least in part, by activation of STAT3 in HL-1 cardiac myocytes exposed to simulated I/R. As shown in Figure 2A, overexpression of STAT3 in HL-1 cardiac myocytes pretreated with Ucn reduced the magnitude of cell death as compared with Ucn treatment alone. To obtain further validation of the above finding, in a similar set of experiments, HL-1 cardiac cells were transfected with a STAT3 mutant containing an alanine residue in place of tyrosine 705. This mutant is functionally inactive and therefore acts as a dominant negative (DN-STAT3). In line with our findings, titrating increasing amounts of the DN-STAT3 enhanced the extent of cell death in a dose-dependent manner in HL-1 cardiac myocytes pretreated with Ucn before exposure to simulated I/R (Fig. 2B).

We subsequently assessed whether the cytoprotective effects of Ucn are also mediated via the Src-pSTAT-T705 phosphorylation pathway. To this end, HL-1 cardiac myocytes overexpressing STAT3 were treated with Ucn in the presence or absence of the Src kinase inhibitor PP2. Figure 2C shows that PP2 reversed the cytoprotective effects of Ucn, as well as the percentage of STAT3 expressing myocytes (data not shown), in a dose-dependent manner. We next overexpressed a constitutively activated form of STAT3, previously shown to mediate cytoprotection, in cardiac cells pretreated with PP2. As shown in Figure 2D and E, the constitutively activated form of STAT3 dose dependently enhanced STAT3 luciferase activity and enhanced cell survival in cardiac myocytes exposed to I/R, despite the presence of PP2. In line with the above findings, by immunocytochemistry, Ucn induced nuclear translocation of pSTAT3-T705, which was inhibited by pretreatment with PP2 (Fig. 3). Together, these data strongly suggest that Ucn can mediate cardioprotection by activating the Src-pSTAT-T705 phosphorylation pathway.

Discussion

In this paper, we show that Ucn treatment of HL-1 cardiac cells results in Src-mediated Tyr 705, but not Ser 727 phosphorylation and activation of STAT3, with a corresponding induction of STAT3 transcriptional activity. Src-mediated STAT3 activation is also required for the cardioprotective effect of Ucn, since a Src inhibitor, and a dominant negative form of STAT3 block Ucn-mediated cardioprotection.

A number of studies have emphasized the important role of STAT3 in cardioprotection following I/R (reviewed in ref. 1). STAT3 has also been implicated in the cardioprotective effects of a variety of agents with known cardioprotective properties, such as resveratrol, and wild-type, but not STAT3 null mesenchymal stem cells promote postischemic myocardial recovery. Moreover, cardiac myocyte specific deletion of STAT3 abolishes ischemic pre- and post-conditioning. In addition, mitochondrial STAT3 partially inhibits electron transport at complexes I and II, resulting in the reduced production of reactive oxygen species and cell death in myocardial I/R injury, though this does not require the phosphorylation or transcriptional activity of STAT3.

However, despite this evidence that acute activation of STAT3 is cardioprotective, sustained STAT3 activation has been implicated in post-infarction remodeling and cardiac hypertrophy. Thus, transgenic mice overexpressing STAT3 in the myocardium show enlarged left ventricles by 12 weeks of age associated with increased expression of hypertrophic genes. Moreover, mice overexpressing both IL-6 and its cognate receptor, which promoted sustained STAT3 activation, also show ventricular hypertrophy. Angiotensin 2 (ATII) is one of the principal mechanisms involved in heart failure following myocardial infarction (reviewed in ref. 25), and ATII signaling through the ATII type I receptor results in delayed STAT3 activation, which may be mediated in part by ATII-induced expression of interleukin-6 (IL-6). Increased IL-6 expression has also been demonstrated in the hypertrophied ventricles of rats with hyperactivity of the ATII system.

The SRC family of tyrosine kinases consists of 9 members (reviewed in ref. 26). It is unclear whether all of these are expressed in cardiac myocytes, though the ancestral cellular member, c-SRC, and LYN and FYN are present. We have previously demonstrated that c-SRC is recruited to CRF receptors in response to Ucn signaling, and is required for the downstream activation of p42/p44 MAPK. C-SRC has also been implicated in ATII-mediated signal transduction. Together, these data suggest that c-SRC-mediated activation of STAT3 following Ucn treatment is involved in the cardioprotective effects of Ucn. Therefore, therapeutic modulation of c-SRC to increase its activity in the acute protective setting and to inhibit it during the sustained hypertrophic environment may be of clinical benefit in ischemic myocardial pathology.

Materials and Methods

Primary cultures of neonatal cardiac myocytes. Ventricular myocytes isolated from the hearts of neonatal Sprague Dawley rats that were less than 2 days old were cultured in Dulbecco’s modified Eagle medium (DMEM) as described previously. To subject cardiac myocytes to simulated ischemia, the normal growth medium of the cardiomyocyte cultures was replaced with 1 ml of ischemic buffer (137 mM NaCl, 12 mM KCl, 0.49 mM MgCl2, 0.9 mM CaCl2H2O, 4 mM HEPES, 20 mM Na lactate, 10 mM
Cell death assay. Apoptosis was assessed by Terminal dUTP nick end labeling (TUNEL). Labeling of 3-hydroxyl ends of DNA fragments was performed using terminal deoxynucleotidyl transferase (TdT) and rhodamine conjugated nucleotides (Roche). Cells were grown on 1% gelatin coated coverslips and transfected for 48 h with 0.25 μg pcDNA3 or STAT3 per well and co-transfected with 0.5 μg GFP to assess the transfection efficiency. Following I/R injury, cells were washed in PBS and deoxyglucose) and the cells were incubated for 4 h in an ischemic chamber containing an atmosphere of virtual 0% oxygen and 5% CO₂. At the end of the ischemic phase, cells were returned to a normoxic environment for a further 16 h of reoxygenation in DMED media, to simulate reperfusion.

Ucn (10⁻⁸ M) was added to cultured myocytes 1 h before hypoxia with or without 5 μM PP2. TOPRO3 was used when required as a counterstain.

**Figure 2.** (A) Overexpression of STAT3 or control (pCMV empty vector) in HL-1 cardiac myocytes pretreated with Ucn reduced the magnitude of cell death as compared with Ucn treatment alone. (B) Increasing amounts of the DN-STAT3 enhanced the extent of cell death in a dose-dependent manner in HL-1 cardiac myocytes pretreated with Ucn before exposure to hypoxiareoxygenation. (C) PP2, a selective inhibitor of the Src family, reversed in a dose-dependent manner the cytoprotective effects of Ucn in HL-1 cells pretreated with Ucn and subsequently exposed to simulated ischemia-reperfusion injury. (D and E) Constitutively activated STAT3 enhanced in a dose dependent fashion STAT3 luciferase activity and enhanced cell survival in cardiac myocytes exposed to simulated ischemia-reperfusion injury despite the presence of PP2. Data represent the mean ± SE of three independent experiments *P < 0.05.
Figure 3. Fluorescent microscopy showing nuclear relocation of phosphorylated-STAT3 (red) in HL-1 cells pretreated with Ucn. Topro-3 was used as a nuclear counterstain (blue).

fixed for 10 min in 4% paraformaldehyde. The TUNEL assay was performed as previously described.21

Luciferase reporter assays. Cells were seeded in 24-well plates and transfected with a 0.5 μg luciferase reporter construct and 50 ng CMV-renilla per well. After 24 h, cells were lysed in passive lysis buffer (Promega) and luciferase activity was measured, using the dual luciferase reporter system according to the manufacturer’s instructions. Relative luciferase activity was calculated as luciferase activity/renilla activity and normalized to controls.

Confocal microscopy. HL1 cells were seeded on glass coverslips and transfected using GeneJuice transfection reagents (Novagen). After fixation in −20 °C methanol, cells were incubated for 60 min in PBS with 3% BSA, followed by incubation for additional 60 min with a mouse primary antibody directed against phospho-STAT3. After three washes in PBS, a secondary antibody (Alexa Fluor 488 donkey anti-mouse) diluted 1:1000 in PBS with 1% BSA was incubated for 30 min. Upon completion of three washes in PBS, coverslips were mounted in Vecta Shield mounting mix containing DAPI (Vector Laboratories) as a counterstain. Images were finally obtained using Zeiss LSM 510 Laser scanning confocal microscope.

Western blot. The phospho-STAT3 at Tyr705 and total STAT3 antibodies were purchased from Cell Signaling Technology. Horseradish peroxidase-conjugated secondary antibodies and rhodamine conjugated secondary antibody were purchased from Dako (Glostrup). Total cellular proteins were prepared from the transfected cell lines by harvesting into 2× Laemmli and addition of 5% β-mercaptoethanol or 1 mM dithiothreitol followed by heating to 95 for 5 min, and then cell debris was removed by centrifugation. Following centrifugation to remove cell debris, protein concentration was assessed using the Bradford assay or Coomassie Blue-stained PAGE.

For western blot analysis, 50–100 ug of cellular proteins were resolved by PAGE on either a 10% or 15% gel at a constant 150 V. Resolved proteins were transferred onto Hybond C membranes by wet transfer method and then incubated with the appropriate primary antibody. Peroxidase-conjugated secondary antibodies were used at dilutions of 1:3000 or 1:2000. Signals were developed using the enhanced chemiluminescence systems (Amersham Biosciences or Pierce).

Statistical analysis. Data are expressed as means ± SEM, all experiments were repeated in triplicate. Statistical analysis was performed using a Student t-test, one-way ANOVA with Dunnet post or two-way ANOVA test with Bonferroni post-test; P values of less than 0.05 were considered significant.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
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