Mitochondrial translocation of Nur77 mediates cardiomyocyte apoptosis

Zhaokang Cheng1†, Mirko Volkers1†, Shabana Din1, Daniele Avitabile1, Mohsin Khan1, Natalie Gude1, Sadia Mohsin1, Tao Bo1, Silvia Truffa1, Roberto Alvarez1, Matt Mason1, Kimberlee M. Fischer1, Mathias H. Konstandin1, Xiao-kun Zhang2, Joan Heller Brown3, and Mark A. Sussman1*

1San Diego State Heart Institute, San Diego State University, North Life Sciences room 426, 5500 Campanile Drive, San Diego, CA 92182, USA; 2Sanford-Burnham Medical Research Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA; and 3Department of Pharmacology, 9500 Gilman Drive, University of California San Diego, La Jolla, CA 92093-0636, USA

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Aims

The cascade of events leading to compromised mitochondrial integrity in response to stress is mediated by various combinatorial interactions of pro- and anti-apoptotic molecules. Nur77, an immediate early gene that encodes a nuclear orphan receptor, translocates from the nucleus to mitochondria to induce cytochrome c release and apoptosis in cancer cells in response to various pro-apoptotic treatments. However, the role of Nur77 in the cardiac setting is still unclear. The objective of this study is to determine the physiological relevance and pathophysiological importance of Nur77 in cardiomyocytes.

Methods and results

Myocardial Nur77 is upregulated following cardiomyopathic injury and, while expressed in the postnatal myocardium, declines in level within weeks after birth. Nur77 is localized predominantly in cardiomyocyte nuclei under normal conditions where it is not apoptotic, but translocates to mitochondria in response to oxidative stress both in vitro and in vivo. Mitochondrial localization of Nur77 induces cytochrome c release and typical morphological features of apoptosis, including chromatin condensation and DNA fragmentation. Knockdown of Nur77 rescued hydrogen peroxide-induced cardiomyocyte apoptosis.

Conclusion

Translocation of Nur77 from the nucleus to the mitochondria in cardiomyocytes results in the loss of mitochondrial integrity and subsequent apoptosis in response to ischaemia/reperfusion injury. Our findings identify Nur77 as a novel mediator of cardiomyocyte apoptosis and warrants further investigation of mitochondrial Nur77 translocation as a mechanism to control cell death in the treatment of ischaemic heart diseases.

Keywords

Nur77 • Mitochondria • Cardiomyocyte • Apoptosis

Introduction

Cardiovascular diseases are the leading cause of death in developed countries and account for almost 40% of all deaths in the USA.1 Acute myocardial infarction (MI) (ischaemia) results in massive myocyte death through necrosis and apoptosis, leading to cardiac dysfunction and sudden death. Early reintroduction of blood flow (reperfusion) reduces the extent of necrosis and rescues part of the ischaemic myocardium, thus remaining the best strategy for reducing infarct size and improving clinical outcome.2 However, myocardial ischaemia/reperfusion (I/R) injury, which is characterized by calcium overload and oxidative stress, initiates the intrinsic apoptotic pathway through activation of the mitochondrial permeability transition pore, a voltage-dependent channel located in the inner mitochondrial membrane.2–4 Reperfusion therapy results in progressive loss of cardiomyocytes, which contributes to heart failure. Although I/R injury is now a well-accepted phenomenon in the clinical setting,5 precise mechanism(s) remain to be elucidated.

The nuclear orphan receptor Nur77 (also known as NR4A1, TR3, and NGFI-B), an immediate-early response gene, is implicated in...
diverse cellular events, such as apoptosis, glucose and lipid metabolism, inflammation, differentiation, survival, and proliferation in various cell types. Nur77 is composed of the N-terminal transactivation domain, central DNA binding domain (DBD), and C-terminal ligand-binding domain. Nur77 induces apoptosis through transcriptional activation of pro-apoptotic genes FasL, TRAIL, NDG1, and NDG2 in T cells, thymocytes, and colon cancer cells. However, recent studies suggest that mitochondrial targeting of Nur77, but not its transcriptional activity, is essential for its pro-apoptotic effect. Nur77 translocates to mitochondria through interaction with Bcl-2, resulting in cytochrome c release and apoptosis by conversion of Bcl-2 from an anti- to pro-apoptotic mediator. However, recent studies suggest that mitochondrial targeting of Nur77, but not its transcriptional activity, is essential for its pro-apoptotic effect. Nur77 translocates to mitochondria through interaction with Bcl-2, resulting in cytochrome c release and apoptosis by conversion of Bcl-2 from an anti- to pro-apoptotic mediator. Although Nur77 has drawn significant attention as a pro-apoptotic molecule in the context of cancer, the role of this molecule in the cardiac setting remains unclear. Here we demonstrate Nur77 translocation to mitochondria during IR injury mediates cardiomyocyte apoptosis. In vitro and in vivo findings indicate that oxidative stress triggers mitochondrial translocation of Nur77, which ultimately results in loss of mitochondrial integrity and release of cytochrome c. Nur77 is a novel mediator of apoptosis in the setting of cardiomyocytes involving both mitochondrial translocation and interaction with normally anti-apoptotic molecules that will be important to further our understanding of how to limit cell death in response to cardiomyopathic injury.

Methods

Animals

All experiments were performed in 8–12-week-old male FVB/N mice unless otherwise indicated. The Institutional Animal Care Committee of San Diego State University approved all animal protocols. Myocardial infarction, ischaemia/reperfusion, and trans-aortic constriction

Surgical procedures were performed as previously described. Briefly, MI was produced by ligating the left anterior descending (LAD) branch of the coronary artery using a 8-0 suture (Ethicon). Ischaemia/reperfusion injury was induced by ligating the LAD for 50 min followed by reperfusion for indicated times. For trans-aortic constriction (TAC), the aorta was ligated between the innominate artery and the left common carotid artery using a 8-0 suture (Ethicon). Ischaemia/reperfusion injury was induced by ligating the LAD for 50 min followed by reperfusion for indicated times. For trans-aortic constriction (TAC), the aorta was ligated between the innominate artery and the left common carotid artery using a 8-0 suture (Ethicon).

Neonatal rat cardiomyocyte cell culture and treatment

Primary neonatal rat cardiomyocytes (NRCM) were isolated from 2- to 3-day-old male and female Sprague–Dawley rat hearts as described previously. Cells were serum-starved in 0.5% FBS for 48 h before treatment with hydrogen peroxide (H2O2, 50 μM or 10 μM, Fisher BioReagents) for the indicated times.

Plasmid construction

Human Nur77 complementary DNA (cDNA) and a Nur77 mutant lacking the DBD (Nur77∆DBD-GFP) were kindly provided by Dr Xiao-kun Zhang (Burnham Institute, San Diego, CA, USA). The Nur77-GFP fusion gene was created by subcloning GFP cDNA into pShuttle-Nur77 plasmid. Schematic diagrams of plasmids used in this study are shown in Supplementary material online, Figure S1.

Adenoviral infection, plasmid, and small interfering RNA transfection

Neonatal rat cardiomyocytes were infected with adenoviruses at multiplicity of infection (MOI) 50 for 2 h and then refed with M199 with 0.5% FBS.

Plasmid transfection of NRCM was performed by using the Effectene transfection reagent (Qiagen). Briefly, 1 μg of DNA was diluted in 100 μL of DNA-condensation buffer followed by 10 μL of Enhancer and 20 μL of Effectene transfection reagent. The transfection complexes were added dropwise onto the cells.

Neonatal rat cardiomyocytes were transfected with small interfering RNAs (siRNAs, 25 nM) by using HiPerfect transfection reagent (Qiagen). Briefly, 3 μL of siRNA and 12 μL of HiPerfect were diluted in 100 μL serum-free M199 medium. After incubation for 5–10 min, transfection complexes were added to the cells for 48 h. The siRNA sequence for rat Nur77 was 5′-UGG CCC AGA GUU CCC UGA AGU UG UU-3′. The scrambled siRNA was obtained from Ambion.

Real-time RT-PCR

Total RNA was isolated from frozen heart or cultured cells by using Quick-RNA™ Miniprep (Zymo Research) and reverse-transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed on all samples in triplicate using QuantiTect® SYBR Green PCR Kit (Qiagen) according to the manufacturer’s instructions. All primer sequences are shown in Supplementary material online, Table S1.

Subcellular fractionation

Subcellular fractionation was performed as described previously. Briefly, heart tissues or NRCM were homogenized in isolation buffer (70 mM sucrose, 190 mM D-Mannitol, 20 mM Hepes, 0.2 mM EDTA). Nuclear fractions were separated by centrifugation at 600g for 10 min followed by discontinuous sucrose density centrifugation. Mitochondrial fractions were separated by centrifugation at 5000g for 15 min. Cytosolic fractions were separated by centrifugation at 100 000 g for 60 min.

Immunoblotting

Protein lysates were loaded onto a 4–12% NuPAGE Novex Bis-Tris Gel (Invitrogen) for electrophoresis. Separated proteins were then transferred onto a polyvinylidene fluoride membrane, blocked with 5% skim milk, and exposed to rabbit anti-Nur77 (sc-5569, Santa Cruz Biotechnology, 1:200), mouse anti-β-actin (sc-7010, Santa Cruz Biotechnology, 1:1000), or mouse anti-GAPDH (MAB374, Chemicon, 1:2000) overnight at 4°C. Alkaline phosphatase, horseradish peroxidase, or Cy5-conjugated IgG (Jackson Immunoresearch, West Grove, PA, USA) were used as secondary antibodies. Fluorescence signal was detected and quantified using a Typhoon 9400 fluorescence scanner together with ImageQuant 5.2 software (Amersham Biosciences).

Immunohistochemistry, TUNEL staining, and confocal microscopy

Cells were permeabilized in 0.1% Triton X-100 for 5 min, blocked with 10% horse serum in PBS for 1 h, and incubated with rabbit anti-Nur77 (LS-B114, Lifespan Biosciences, 1:50), goat anti-heat shock protein 60 (HSP60, sc-1052, Santa Cruz Biotechnology, 1:50), mouse anti-cytochrome c (S56432, BD Pharmingen, 1:50), mouse anti-α-actinin (A7732, Sigma-Aldrich, 1:100) at 4°C overnight. The next day, slides were incubated 1.5 h at room temperature with...
FITC, Cy3, or Cy5-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, 1:100). Nuclei were stained with Topro-3 iodide or Sytox Blue (Invitrogen).

Formalin-fixed paraffin-embedded mouse heart sections were deparaffinized, rehydrated, and antigen-retrieved in 10 mmol/L citrate, pH 6.0. After blocking in TNB buffer, slides were incubated with rabbit anti-Nur77 (LS-B114, Lifespan Biosciences, 1:100) and mouse anti-tropomyosin (T9283, Sigma-Aldrich, 1:100) at 4°C overnight. Confocal images were acquired by using a Leica TCS-SP2 or a Molecular Dynamics CLSM 2010 confocal laser-scanning microscope (Leica).

TUNEL staining was performed by using the In Situ Cell Death Detection Kit, TMR red (Roche Applied Science). Briefly, NRCM were fixed in 4% PFA and permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate in PBS. Cells were then incubated with TUNEL reagent for 1 h at 37°C and covered for examination by confocal microscopy.

Flow cytometry for cell death
Cells were stained with Annexin V (1:50, BD Biosciences) and Sytox Blue (1:1000, Invitrogen) for 15 min. Annexin V binds exposed membrane phospholipid phosphatidylserine during early apoptosis, whereas Sytox Blue is a high-affinity nucleic acid stain that easily penetrates damaged cell membranes. Early apoptotic cells are Sytox Blue−/Annexin V+; late apoptotic cells are Sytox Blue+/Annexin V+; necrotic cells are Sytox Blue+/Annexin V−; and viable cells are Sytox Blue−/Annexin V−. Flow cytometry was performed using a BD FACS Aria Flow Cytometer (BD Biosciences).

Statistical analysis
Statistical analysis was performed with the Windows SPSS 13.0 software package. All data are expressed as mean ± SEM. Comparisons were performed by using unpaired Student’s t-test or one-way analysis of variance with Tukey’s post hoc test as appropriate. All tests were two-sided and a value of P < 0.05 was considered statistically significant.

Results
Myocardial Nur77 declines during postnatal development
Mice at various postnatal ages were sacrificed and hearts were processed for immunohistochemistry or immunoblotting. Intracellular localization of Nur77 was examined in myocardial sections. Nur77 localizes predominantly to the nuclei rather than cytoplasm of cardiomyocytes by confocal microscopy (Figure 1A). Nur77 expression is higher in the neonatal heart, decreasing by ~60% in the adult heart by immunoblot analyses (Figure 1B). Transcript levels of Nur77 in the adult heart are approximately 10% of that in the neonatal heart (Supplementary material online, Figure SII). Nuclear localization of Nur77 was also confirmed by immunofluorescence staining of cultured NRCM (Figure 1C).

Figure 1 Myocardial Nur77 expression declines during postnatal development. (A) Confocal images of mice hearts at 2 days, 7 days, and 2 months after birth immunolabelled for Nur77 (green), Topro-3 (blue), and tropomyosin (red). Nur77 is predominately nuclear. Boxed regions in left are shown at higher magnification at right. Scale bar = 40 μm (left) and 10 μm (right); (B) decline of the myocardial Nur77 protein during postnatal development assessed by immunoblot of ventricle lysates from mice at ages of 2 days, 7 days, 14 days, 2 months, and 1 year, with β-actin as a loading control (n = 3). **P < 0.01 vs. 2 days, ***P < 0.001 vs. 2 days; (C) confocal microscopy of Nur77 expression in cultured neonatal rat cardiomyocytes showing nuclear localization. Scale bar = 20 μm.
Nur77 is upregulated after myocardial infarction and hypertrophy

Nur77 localization and expression were examined in two murine models for myocardial injury: induced MI and pressure overload-induced hypertrophy by TAC. Following MI, Nur77 is localized predominantly in cardiomyocyte nuclei residing within the border zone (Supplementary material online, Figure SIIIA). Nur77 levels are elevated subsequent to MI challenge, peak at 3 days, and decrease thereafter as shown by immunoblot assessment of myocardial lysates (Supplementary material online, Figure SIIIB). Cardiomyopathic insult by TAC produced marked immunoreactivity for Nur77 in cardiomyocyte nuclei at 4 days after challenge (Supplementary material online, Figure SIIID). In agreement with protein expression findings, TAC also induced upregulation of Nur77 mRNA in conjunction with hypertrophy markers: atrial natriuretic peptide, brain natriuretic peptide, and β-myosin heavy chain (Supplementary material online, Figure SIIIE).

Mitochondrial translocation of Nur77 is induced by ischaemia/reperfusion in vivo

Nur77 translocation to mitochondria is associated with apoptosis.16 Subcellular localization and levels of Nur77 during IR were measured to confirm Nur77 translocation to mitochondria in cardiomyocytes following pathological challenge. Hearts were subjected to 50 min of ischaemia followed by 15 min and 120 min of reperfusion. In the sham-operated heart, Nur77 localizes predominately in nuclei of cardiomyocytes. In comparison, marked colocalization of Nur77 with mitochondria (as identified by HSP60) was present after 50 min of ischaemia and 120 min of reperfusion (Figure 2A). Protein levels of Nur77 were not significantly changed between sham-operated vs. reperfused heart samples (Figure 2B). However, the presence of nuclear Nur77 is decreased after 120 min reperfusion (Figure 2C), whereas cytosolic and mitochondrial Nur77 are significantly increased at this time point (Figure 2D and E). These effects on Nur77 translocation are dependent upon reperfusion stimulation, since samples from hearts subjected to comparable ischaemic treatment without reperfusion (i.e. 65 and 170 min) show no changes in total Nur77 level or intracellular localization (Supplementary material online, Figure SIV). Staining of the myocardial sections for oxidative DNA damage confirmed ROS activity after IR (Supplementary material online, Figure SV). Thus, Nur77 translocates from nuclei to cytosol and mitochondria in response to IR injury.

Mitochondrial translocation of Nur77 is induced by oxidative stress in vitro

Oxidative stress plays a critical role in IR-induced cardiomyocyte apoptosis.3 To test whether oxidative stress induces mitochondrial translocation of Nur77, NRCM were exposed to 50 μM H$_2$O$_2$ and immunostained for Nur77. Before the H$_2$O$_2$ challenge, Nur77 localizes primarily in nuclei (Figure 3A), but colocalization of Nur77 with the mitochondrial marker HSP60 appears at 30 and 60 min following treatment (Figure 3A). In accordance, Nur77 localizes in the nucleus of adult cardiomyocytes and translocates into the cytosol and mitochondria 30 min following treatment with 50 μM H$_2$O$_2$. Corroborating results were obtained from NRCM subjected to identical H$_2$O$_2$ challenge and subsequent subcellular fractionation. While the level of total Nur77 protein remains unchanged by H$_2$O$_2$ treatment (Figure 3C), the presence of Nur77 in the nuclear fraction is significantly decreased (Figure 3D) with concomitant increases in cytosolic (Figure 3E) and mitochondrial Nur77 (Figure 3F). Translocation of Nur77 from nucleus to cytosol and mitochondria prompted by oxidative stress was confirmed using an adenovirally encoded Nur77-GFP fusion product. Nur77-GFP localized exclusively to NRCM nuclei under normal culture conditions, with H$_2$O$_2$ treatment prompting mitochondrial translocation (Figure 3G).

Mitochondrial targeting of Nur77-induced cardiomyocyte apoptosis

A Nur77 mutant lacking the DBD (Nur77/ΔDBD) localizes at mitochondria to induce apoptosis in cancer cells.16 These results were confirmed via transient transfection with expression plasmids for GFP, Nur77-GFP, or Nur77/ΔDBD-GFP in 293T cells stained with Sytox Blue and Annexin V. Early apoptotic (Sytox Blue−/Annexin V+), late apoptotic (Sytox Blue−/Annexin V−), and necrotic (Sytox Blue+/Annexin V−) cells are considered dead cells in this assay. Nur77/ΔDBD-GFP expression markedly increases cell death compared with either GFP alone or the Nur77-GFP fusion protein (Figure 4A and B). Importantly, the expression level of Nur77/ΔDBD was similar to the wild-type protein (Figure 4C). Cytopathic effects of the Nur77/ΔDBD-GFP construct prevented creation of a recombinant adenoviral expression vector in our hands, but conventional transfections using the Nur77-GFP or Nur77/ΔDBD-GFP plasmids were performed using NRCM. Nur77/ΔDBD-GFP, but not Nur77-GFP, appeared in mitochondria and induced cytochrome c release (Figure 4D; at arrow). Pro-apoptotic effects of the Nur77/ΔDBD-GFP construct were confirmed by colocalization of nuclei with the TUNEL signal. Nur77/ΔDBD-GFP-transfected NRCM show typical morphological features of apoptosis (Figure 4E), including chromatin condensation (arrows) and DNA fragmentation (arrowheads) that were not observed in the control Nur77-GFP-transfected cells. Quantitative analysis confirms that Nur77/ΔDBD-GFP significantly increased TUNEL-positive NRCM compared with Nur77-GFP (Figure 4F).

Knockdown of Nur77 attenuated oxidative stress-induced apoptosis

Participation of Nur77 in apoptotic cell death induced by oxidative stress was determined in NRCM transiently transfected with scrambled siRNA or Nur77-specific siRNA (si-Nur77). Nur77 expression is significantly reduced by Nur77 siRNA 48 h after transfection by immunoblot analysis (Figure 5A) and Nur77 fluorescence intensity was also noticeably decreased by si-Nur77 (Figure 5B). These si-Nur77-transfected NRCM were subsequently challenged with 50 μM H$_2$O$_2$ for 1 h or 10 μM H$_2$O$_2$ for 5 h. Reduction of Nur77 by this siRNA approach corresponds with a significant attenuation of H$_2$O$_2$-induced apoptosis as measured by TUNEL labelling (Figure 5C and D) as well as flow cytometry...
using Annexin V and Sytox Blue to identify viable cells (Figure 5E). Thus, Nur77 plays a pro-apoptotic role in oxidative stress-induced cell death in cultured NRCM.

**Discussion**

Although Nur77 was first described over two decades ago, its role in cardiac biology is still unclear. Nur77 predominates in the nuclear subcellular compartment under normal conditions and exerts cytopathic effects upon translocation from the nucleus to mitochondria. Cardiac Nur77 decreases during postnatal development but is induced by pathological injury including MI and pressure overload-induced hypertrophy. Moreover, reperfusion injury in the intact myocardium known to provoke oxidative stress correlates with Nur77 translocation to mitochondria. These findings correlate with apoptotic cell death from...
mitochondrial accumulation of mutant Nur77 lacking DNA binding capacity in NRCM and 293T cells. Although Nur77-induced cytochrome c release by translocation to mitochondria in cardiomyocytes was previously described in rats stressed by restraint, the presence of nuclear Nur77 under control conditions was not addressed.22 In our study, oxidative stress appears as a primary mediator of Nur77 mitochondrial translocation both in vivo (Figure 2) and in vitro (Figure 3).

Nur77 localizes predominantly in the nuclei of cardiomyocytes under normal conditions. We noticed that Nur77 expression alone does not provoke apoptotic cell death as evidenced by our adenoviral overexpression experiments with normal Nur77, which failed to show a significant increase in TUNEL-positive NRCM. Rather, the localization to mitochondria, induction of cytochrome c release, and apoptosis of NRCM are induced by expression of a Nur77/ΔDBD-GFP mutant lacking DNA binding capabilities (Figure 4). The Nur77 protein sequence contains two nuclear localization signals (NLS1: KRRRNR at residues 278–283; and NLS2: KGRRGR at residues 308–313), which are localized in the DNA-binding domain of Nur77.23 Mutation of NLS resulted in diffuse distribution of Nur77 throughout the cells.23 While both endogenous and exogenous Nur77 are observed predominantly in NRCM nuclei during normal conditions (Figure 3A and G), Nur77/ΔDBD-GFP was diffusely distributed and localized to mitochondria (Figure 4D) due to deletion of the NLS region. In PC12 cells, nuclear export of Nur77 requires presence of nuclear export signals (NES). The Nur77 protein sequence also contains three leucine-rich NES with mutation of the NES disrupting Nur77 nuclear export.23 Nur77 nuclear export is mediated by CRM1 (also known as exportin 1), which binds NES regions and initiates nuclear export in various cell types.16,23–25

The Nur77 protein lacks classical mitochondrial localization signals, but can localize to mitochondria through interaction with Bcl-2.17 The Bcl-2 N-terminal loop region, located between BH4 and BH3 domains, plays a critical role in regulating Bcl-2 functions.26 Phosphorylation, deamidation, or cleavage within the loop region decrease the anti-apoptotic activity of Bcl-2 and enhance pro-apoptotic action.12,24 The ligand-binding domain of Nur77 interacts with the Bcl-2 loop region, resulting in
displacement of its BH4 domain and exposure of the BH3 death domain, which finally leads to mitochondrial disruption and apoptosis.\textsuperscript{17,18} Interestingly, another pro-apoptotic transcription factor p53 also translocates to mitochondria and interacts with Bcl-2/Bcl-XL, resulting in transcription-independent apoptosis.\textsuperscript{27,28}

Mitochondrial translocation of Nur77 is essential for pro-apoptotic action as observed during IR \textit{in vivo} or H\textsubscript{2}O\textsubscript{2}-induced oxidative stress \textit{in vitro} (Figure 6). In agreement with our data, oxidative stress-induced translocation of Nur77 to mitochondria is crucial in neuronal cell death \textit{in vivo}.\textsuperscript{24} In the current study, cardiomyocyte survival/apoptosis was not affected by increasing (Figure 4D) or decreasing (Figure 5D) the level of nuclear Nur77 under normal conditions. For the \textit{in vitro} transfection and knockdown experiments, we used neonatal instead of adult cardiomyocytes because neonatal myocytes can be cultured for long periods of time without the loss of viability. In adult cardiomyocytes, Nur77 also translocated to mitochondria in response to oxidative stress both \textit{in vivo} (Figure 2) and \textit{in vitro} (Figure 3B), indicating that neonatal and adult myocytes behaved similarly at least in these respects. Our observation that neonatal and pathologically-challenged cardiomyocytes express higher levels of Nur77 may explain why these cells are more vulnerable to environmental stimuli.

From a therapeutic standpoint, our results may have clinical implications for the treatment of ischaemic heart diseases and heart failure. Prevention of mitochondrial Nur77 localization represents a novel approach to inhibit cardiomyocyte apoptosis. Indeed, the nuclear export inhibitor Leptomycin B (LMB) has been shown to prevent apoptosis of thymocytes\textsuperscript{19} and various cancer cells\textsuperscript{29–31} by blocking Nur77 cytoplasmic localization. However, cytotoxicity of LMB limits potential clinical application.\textsuperscript{32} Therefore, Nur77-specific nuclear export inhibitors with high...
potency and low toxicity would need to be explored to take advantage of this cytoprotective approach.

Clear ties between cardioactive signalling cascades and the actions of Nur77 are implied from studies in the non-cardiac context. Multiple cardioprotective signalling pathways influence Nur77 by post-translational modification. Phosphorylation by Akt reduces transcriptional activity of Nur77, connecting nuclear Akt activity to this orphan nuclear receptor pathway. Additionally, nuclear export of Nur77 was linked to c-Jun N-terminal kinase (JNK) activation and inhibition of Akt. Activation of extracellular signal-related kinase 2 (ERK2) inhibits the subcellular translocation and subsequent apoptosis associated with Nur77 mitochondrial accumulation that may help account for ERK2-mediated cardioprotection reported to be required for blunting of IR injury.

**Figure 5** Knockdown of Nur77 inhibits cardiomyocyte apoptosis. Scrambled or Nur77 siRNA transfected into neonatal rat cardiomyocytes (NRCM) then treated with 50 μM H₂O₂ for 1 h or 10 μM H₂O₂ for 5 h. (A) Immunoblot shows decreased Nur77 level by >70% with Nur77 siRNA transfection. **P < 0.01. (B) Immunolabelling shows decreased Nur77 signal intensity (green) by si-Nur77 transfection. Nuclei stained with Topro-3 (blue). (C) TUNEL label of H₂O₂-treated cells showing that knockdown of Nur77 decreased TUNEL-positive cells (red) with nuclei (green). Colocalization of TUNEL signal and nuclei appears yellow (at arrows). (D) Quantitative analysis of TUNEL staining shows knockdown of Nur77 inhibited H₂O₂-induced NRCM apoptosis (n=4). *P < 0.05, **P < 0.01. (E) Flow cytometry shows knockdown of Nur77 protects NRCM from H₂O₂-induced cell death (n=3). Cells treated with 10 μM H₂O₂ for 5 h and stained for Annexin V and Sytox Blue, with viability determined as Sytox Blue/Annexin V−. Data expressed as fold change vs. non-treated control. *P < 0.05, ***P < 0.001.
in vivo. Additional modifications in the form of acetylation/deacetylation by p300 and HDAC1, respectively, are also reported to regulate Nur77 protein turnover. Associations between Nur77 and calcium-dependent signalling as well as creation of Nur77 knockout mice occurred over a decade ago, but the search for clear phenotypic effects continues at present. Initial studies of Nur77 knockout mice have produced no overt phenotype and the presumption is that functional redundancy must exist, but the connection between Nur77 and mitochondrial translocation had yet to be made in these early studies. Around the same time, DNA binding activity of Nur77 was observed to be cyclosporine-sensitive, implicating a role for calcium-dependent signalling in Nur77-mediated regulation of thymocyte survival. Now 15 years later, a combination of calcium influx and protein kinase C activation has been found to induce mitochondrial targeting of Nur77 in thymocytes. Collectively, these studies demonstrate that there is still much to discover regarding the role of Nur77 in cell survival and regulation of this orphan nuclear receptor by signal transduction. Our study sets the stage for future investigations of Nur77 biology in the myocardial context.

Supplementary material
Supplementary material is available at European Heart Journal online.

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