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The cardioprotective effect of necrostatin requires the cyclophilin-D component of the mitochondrial permeability transition pore

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Abstract

Necrostatin (Nec-1) protects against ischemia-reperfusion (IR) injury in both brain and heart. We have previously reported in this journal that necrostatin can delay opening of the mitochondrial permeability transition pore (MPTP) in isolated cardiomyocytes. The aim of the present study was to investigate in more detail the role played by the MPTP in necrostatin-mediated cardioprotection employing mice lacking a key component of the MPTP, namely cyclophilin-D. Anaesthetized wild type (WT) and cyclophilin-D knockout (Cyp-D^{-/-}) mice underwent an open-chest procedure involving 30 minutes of myocardial ischemia and 2 hours of reperfusion, with subsequent infarct size assessed by triphenyltetrazolium staining. Nec-1, given at reperfusion, significantly limited infarct size in WT mice (17.7±3% vs. 54.3±3%, $P<0.05$) but not in Cyp-D^{-/-} mice (28.3±7% vs. 30.8±6%, $P>0.05$). In conclusion, the data obtained in Cyp-D^{-/-} mice provide further evidence that Nec-1 protects against myocardial IR injury by modulating MPTP opening at reperfusion.

Keywords

Necrostatin; mitochondrial permeability transition pore; cyclophilin-D

Introduction

Necrostatin (Nec-1), a tryptophan-based compound, has been proposed to be a selective blocker of a putative form of cell death distinct from necrosis and apoptosis termed necroptosis.[1] The cytoprotective effect of Nec-1 was first demonstrated by Degterev and colleagues employing a stroke model in which intracerebroventricular injection of Nec-1 was found to protect neurons against brain ischemia.[1] We recently reported that Nec-1 also protects the heart in various experimental systems, including cardiac myocyte cell culture and *in vitro* and *in vivo* models of myocardial ischemia-reperfusion (IR) injury.[2] However, to date, little information is available concerning the cellular mechanisms underlying necroptosis or the processes by which Nec-1 produces its action, although the possibility that Nec-1 functions as an antioxidant has been discounted.[1;2] We have demonstrated that Nec-1 can delay the opening of the mitochondrial permeability transition pore (MPTP) in isolated rat cardiomyocytes subjected to oxidative stress,[2] but whether this is the mechanism by which it causes cardioprotection is not known. The MPTP is a non-specific channel of the inner mitochondrial membrane, the opening of which in the first few

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minutes of myocardial reperfusion mediates cell death by uncoupling oxidative phosphorylation and inducing mitochondrial swelling.[3;4] Inhibiting MPTP opening with pharmacological inhibitors such as cyclosporin-A and sanglifehrin-A at reperfusion, has been demonstrated to protect the heart against IR injury.[4-6] Although the structure of the MPTP is currently unclear, recent studies have provided convincing evidence that cyclophilin-D (Cyp-D) is an important regulatory component of the MPTP, such that mice deficient in Cyp-D are resistant to MPTP opening and sustain both smaller myocardial and cerebral infarcts in response to IR injury.[7-10] In addition, we have also demonstrated that ischemic preconditioning and postconditioning both require the Cyp-D component of the mPTP in order to protect the heart *in vivo*. [11] Furthermore, a range of well-established cardioprotective agents including bradykinin and diazoxide appear to all require Cyp-D.[11] Given that necrostatin has been proposed to act by inhibiting a distinct mode of cell death (i.e. "necroptosis"),[1] we hypothesized that it would have cardioprotective activity even in the absence of Cyp-D. Therefore, the present study was undertaken to test this hypothesis in Cyp-D^{-/-} mice that are incapable of forming a functional MPTP.

Methods

Animals

Experiments using animals were carried out in accordance with the United Kingdom Home Office Guide on the Operation of Animal (Scientific Procedures) Act of 1986. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. B6129SvF1 wild type (WT) mice were obtained from Harlan (UK) and Cyp-D^{-/-} mice were generated as previously described.[8]

In vivo model of ischemia reperfusion injury

WT and Cyp-D^{-/-} mice (male, 8-10 weeks, 20-30 g) were anesthetized (ketamine/xylazine/atropine). The external jugular vein and carotid artery were cannulated for drug administration and mean arterial blood pressure measurement, respectively. A tracheotomy was performed for artificial respiration at 120 strokes/min and 220 ~1 stroke volume using a rodent Minivent (type 845, Harvard Apparatus, Kent, UK) and supplemental oxygen was supplied. A limb lead I electrocardiogram (ECG) was recorded. A thoracotomy was then performed and the left anterior descending (LAD) coronary artery was ligated ~2 mm below the tip of the left auricle using a 8/0 prolene monofilament polypropylene suture. Successful LAD coronary artery occlusion was confirmed by the presence of ST elevation and a decrease in arterial blood pressure. Mice were subjected to 30 minutes of ischemia followed by 120 minutes of reperfusion, and the infarct size and area at risk (AAR) were determined by Evan's blue and triphenyltetrazolium chloride dual-staining. AAR was expressed as a percentage of the left ventricle and infarct size was expressed as a percentage of the AAR[11] A bolus dose of Nec-1 (1.65 mg/kg) or vehicle control (0.2% DMSO) was administered intravenously at the onset of reperfusion.

Chemicals

Nec-1 was purchased from Calbiochem (La Jolla, USA), heparin from CP Pharmaceuticals Ltd (Wrexham, UK), ketamine (Vetalar®) from Pharmacia Animal Health Ltd (Northamptonshire, UK), SfA from Novartis Pharma (Basel, Switzerland) and xylazine (Rompun®) from Bayer Plc. (Berkshire, UK). All other chemicals were purchased from Sigma-Aldrich (Dorset, UK). Nec-1 was dissolved in DMSO (final concentration 0.2%).

Statistical Analysis

All values are expressed as mean $\pm$ SEM. Data were analyzed by One-way ANOVA followed by a Tukey's multiple comparison post-hoc test. A $P < 0.05$ was considered to be statistically significant.

Results

The area at risk (AAR) was comparable among the treatment groups (figure 1a). In WT mice, necrostatin, given at reperfusion, significantly reduced infarct size to $17.7 \pm 3\%$ versus $54.3 \pm 3\%$ in vehicle control ($P < 0.05$; figure 1b). As expected, Cyp-D $^{-/-}$ mice exhibited a smaller infarct size ($30.8 \pm 6\%$, $P < 0.05$). Necrostatin, however, did not reduce infarct size in the Cyp-D $^{-/-}$ mice ($28.3 \pm 7\%$ vs. $30.8 \pm 6\%$ in Cyp-D $^{-/-}$ control, $P > 0.05$; figure 1b).

Discussion

The results presented here demonstrate that the Cyp-D, a regulatory component of the MPTP, is required for Nec-1 to exhibit a cardioprotective effect in mice *in vivo*. In wild-type mice Nec-1 reduced infarct size substantially *in vivo* when administered intravenously at the onset of reperfusion but it failed to produce a similar effect in Cyp-D $^{-/-}$ mice.

Previously we have shown that the hearts of Cyp-D $^{-/-}$ mice are not susceptible to ischemic and pharmacological preconditioning and postconditioning.[11] This current study suggests that another recently described cardioprotective agent, albeit one that was proposed to act via a novel death pathway, also functions through the same established mechanisms involving Cyp-D and the MPTP. However, the mechanism by which Nec-1 inhibits MPTP opening remains to be elucidated. It has been postulated that MPTP inhibition occurs as a consequence of activation of the so-called Reperfusion Injury Salvage Kinase (RISK) pathway,[12] which incorporates the pro-survival kinases, phosphatidylinositol 3-OH kinase (PI3K)-cellular Akt/protein kinase B (Akt) and p44/42 mitogen-activated protein kinase (MAPK) extracellular signal-regulated MAPK (Erk1/2). The RISK pathway appears to form the mechanistic basis of the various cardioprotective interventions described i.e. ischemic and pharmacological preconditioning and postconditioning and MPTP inhibition. [12] Future studies will focus on the relationship between necrostatin and these cell signalling pathways.

In conclusion, the present study has provided confirmatory evidence that Nec-1 protects against acute myocardial IR injury and it acts via Cyp-D preventing MPTP opening at reperfusion.

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Figure 1.

Reduction in infarct size (IS) induced by necrostatin (1.65 mg/kg) *in vivo*. (A) Area at risk (AAR) expressed as a percentage of the left ventricle (LV), and (B) Infarct size expressed as a percentage of the AAR. Data are given as mean $\pm$ SEM with * indicates $P < 0.05$ vs. wild type DMSO vehicle control (n = 5-6).