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Necrostatin: A Potentially Novel Cardioprotective Agent?

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Abstract
Background Necrostatin-1 (Nec-1), a small tryptophan-based molecule, was recently reported to protect the cerebral cortex against ischemia-reperfusion (I/R) injury. We investigated the actions of Nec-1 and its so-called inactive analog, Nec-1i, in the setting of myocardial I/R injury.
Materials and methods The actions of Nec-1 and Nec-1i were examined in cultured C2C12 and H9c2 myocytes, cardiomyocytes isolated from male Sprague–Dawley rats, Langendorff isolated perfused C57Bl/6J mouse hearts and an in vivo open-chest C57Bl/6J mouse heart model.
Results Nec-1 at 30 μM and 100 μM (but not 100 μM Nec-1i) reduced peroxide-induced cell death in C2C12 cells from 51.2±1.1% (control) to 26.3±2.9% ($p<0.01$ vs control) and 17.8±0.9% ($p<0.001$), respectively. With H9c2 cells cell death was also reduced from 73.0±0.4% (control) to 56.7±0% (30 μM Nec-1, $p<0.05$) and 45.4±3.3% (100 μM Nec-1, $p<0.01$). In the isolated perfused heart Nec-1 (30 μM) reduced infarct size (calculated as a percentage of the risk area) from 48.0±2.0% (control) to 32.1±5.4% ($p<0.05$). Nec-1i (30 μM) also reduced infarct size (32.9±5.1%, $p<0.05$). In anesthetized C57Bl/6J mice Nec-1 (1.65 mg/kg), given intraperitoneally to coincide with reperfusion following left anterior descending artery ligation (30 min), also reduced infarct size from 45.3±5.1% (control) to 26.6±4.0% ($p<0.05$), whilst Nec-1i (1.74 mg/kg) was ineffective (37.8±6.0%). Stimulus-induced opening of the mitochondrial permeability transition pore (MPTP) in rat cardiomyocytes, as reflected by the time until mitochondrial depolarisation, was unaffected by Nec-1 or Nec-1i at 30 μM but increased at 100 μM i.e. 91% ($p<0.05$ vs control) and 152% ($p<0.001$) for Nec-1 and Nec-1i, respectively.
Conclusion This is the first study to demonstrate that necrostatins inhibit myocardial cell death and reduce infarct size, possibly via a mechanism independent of the MPTP.

Key words necrostatin · cardioprotection · infarct size · mitochondrial permeability transition pore

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Introduction

Myocardial ischemia-reperfusion (I/R) injury involves two key mechanisms, namely necrosis and apoptosis, the former representing a non-specific, unregulated form of cell death, whilst the latter is a highly regulated, genetically determined process designed to systematically dismantle damaged cells and maintain homeostasis [1]. Protection against I/R injury is mediated through a complex series of events involving the activation of specific cell signalling cascades and resulting ultimately in the generation of reactive oxygen species (ROS) and actions on mitochondrial energy metabolism [2]. In addition we have identified the so-called reperfusion injury salvage kinase (RISK) pathway, incorporating phosphatidyl-inositol 3-OH kinase (PI3K)-cellular Akt/protein kinase B (Akt) and p44/42 mitogen-activated protein kinase (MAPK) extracellular signal-regulated MAPK (Erk 1/2), coupled with inhibition of the mitochondrial permeability transition pore (MPTP) as playing a major role in reperfusion induced injury [3, 4].

Various chemically diverse substances have been shown to protect against myocardial I/R injury, as indicated by reduced infarct size. These substances include drugs such as atorvastatin [5] and pioglitazone [6]. Endogenous factors, for example bradykinin [7], insulin [8], erythropoietin [9], glucagon-like peptide 1 [10] and leptin [11], have also been shown to produce comparable beneficial effects. Recently, Degterev et al. [12] reported that screening of a chemical library of some 15,000 compounds for inhibitors of necrotic cell death resulted in the identification of a small tryptophan-based molecule, termed necrostatin-1 (Nec-1), that reduced infarct volume after middle cerebral artery occlusion following intracerebroventricular injection In addition, a so-called inactive form (as designated by Degterev et al. [12]) of the compound (Nec-1i), which differs from Nec-1 by a single methyl group, was found to produce only marginal decreases (see Fig. 1 for chemical structures of Nec-1 and Nec-1i). The same authors claimed that Nec-1 produced its actions via inhibition of a form of cell death distinct from necrosis and apoptosis which they termed necroptosis [12]. More recently sitosterol-induced death in macrophages was reported to be caspase-independent and blocked by Nec-1, and again it was suggested that necroptosis (and autophagy) might be involved [13], although considerably more work will be required before necroptosis can be unequivocally confirmed as representing a distinct entity.

Protection against I/R injury in the heart and the brain could involve similar mechanisms and this could extend to the particular cell-signaling pathways or enzymes that are activated or down-regulated during the recovery process [14]. As Nec-1 has been shown to limit cellular damage in the brain we decided to ascertain whether it could produce a similar action in the myocardium. To investigate this we used a variety of in vitro experimental models. Thus, the effects of Nec-1 and Nec-1i were investigated in three cellular systems, namely on C2C12 and H9c2 necrotic cell death induced by tert-butyl hydroperoxide (t-BuOOH), C2C12 and H9c2 myoblasts representing accepted models for investigating putative myocardial mechanisms and in the case of the latter being derived from rat heart, as well as on ROS-induced MPTP opening in ventricular cardiomyocytes. The influence of the compounds on I/R-induced injury (as determined by infarct size assessment) in a mouse model of myocardial infarction was also investigated. Finally, in order to replicate as far as is possible the pathophysiological changes occurring in vivo following myocardial infarction and to gain a greater insight into the workings of necrostatin a murine in vivo model of I/R injury was also employed.

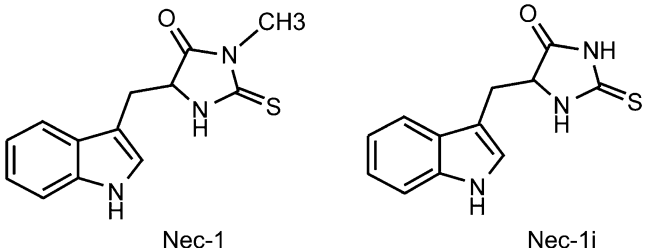

Fig. 1 Chemical structures of Necrostatin-1 (Nec-1) and its "inactive" analogue necrostatin-1i (Nec-1i)

Materials and methods

Animals The current study was carried out in accordance with *The Guide for the care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996) and was approved by the UCL Hospitals Ethics Committee.

Drugs/chemicals Nec-1 and Nec-1i were purchased from Calbiochem whilst other agents came from Sigma Chemical Co.

C2C12 and H9c2 cells C2C12 and H9c2 cells were cultured overnight in 24 well plates at 37°C in MEM containing 10% foetal calf serum (FCS) and 1% penicillin/streptomycin, and gassed with 5% CO_2. On the day of experimentation cells were treated with t-BuOOH (100 μM with H9c2 cells and 200 μM with C2C12 cells) in the presence of DMSO (0.02% final concentration, control incubations) or Nec-1 (3, 10, 30 or 100 μM), Nec-1i (100 μM) or the vitamin E analogue, Trolox (100 μM), for 4 h in the case of H9c2 cells and 5.5 h for C2C12 cells. After incubation the medium was removed and the cells washed with phosphate-buffered saline (PBS) before their removal from the plates by trypsinization. The cells were then transferred to PBS containing 0.1% FCS and 1 μg/ml propidium iodide (PI) contained in tubes on ice. The percentage of dead cells (as indicated by red fluorescence due to PI uptake) was established using a Partec PAS flow cytometer.

Rat cardiomyocytes Ventricular cardiomyocytes were prepared from adult male Sprague–Dawley rat hearts [15]. Cardiomyocyte mitochondrial permeability transition pore (MPTP) opening was monitored employing a model of oxidative stress [15, 16]. Thus, cardiomyocytes were loaded with the fluorescent dye tetra-methyl rhodamine methyl ester (TMRM) and then underwent laser stimulation which results in mitochondrial ROS generation (i.e. simulating ROS generation during reperfusion) with subsequent MPTP opening. MPTP opening was reflected by mitochondrial depolarisation and dequenching of TMRM fluorescence, the time (in seconds) until depolarisation being recorded. In experiments with Nec-1 or Nec-1i, cardiomyocytes were preincubated for 10 min with saline before incubation

(15 min) with the test compounds and subsequent laser stimulation.

Langendorff isolated perfused mouse heart model C57Bl/6J mice were given 100 U of heparin by intraperitoneal injection prior to cervical dislocation. Hearts were then excised and perfused retrogradely via the aorta at constant pressure (100 mmHg) with oxygenated Krebs–Henseleit buffer, myocardial temperature (thermal probe) and heart rate (ventricular balloon) being monitored throughout experiments [5, 17]. Hearts underwent 30 min stabilisation followed by 35 min global ischemia (achieved by total perfusion arrest) and 35 min reperfusion. Krebs buffer containing Nec-1 (30 or 100 μM) or Nec-1i (100 μM) was substituted for normal buffer at reperfusion in some experiments. Following reperfusion hearts were injected with 1% triphenyl-tetrazolium chloride (TTC) and incubated in TTC (37°C, 10 min) before freezing (−20°C). At the time of analysis hearts were sliced (<1 mm slices), destained (formalin), photographed and planimetered (NIH Image 1.63 software package; National Institutes of Health, USA), and infarct size expressed as the percentage of risk volume (I/R %).

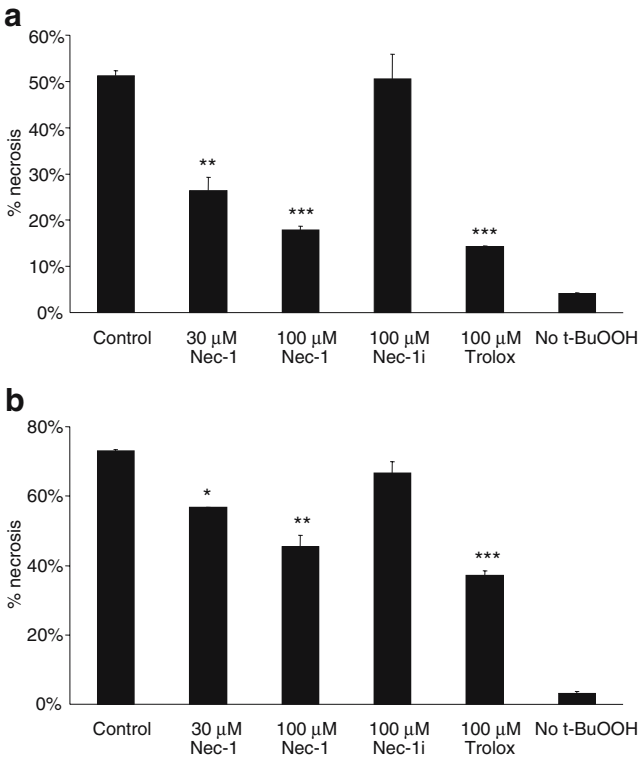

Fig. 2 **a** Suppression by necrostatin of necrotic cell death in C2C12 cells. Cells were treated with t-BuOOH (200 μM) in the presence of DMSO (vehicle, 0.02%), necrostatin-1 (Nec-1; 30 or 100 μM), its "inactive" analogue necrostatin-1i (Nec-1i; 100 μM) or Trolox (100 μM) and cell death assessed by flow cytometry using the fluorescent probe propidium iodide (PI). **b** Inhibition of cell death in H9c2 cultured cells. The experimental conditions were essentially the same as for experiments with C2C12 cells except that necrosis was induced by 100 μM t-BuOOH. Data are presented as mean±SEM (n=4) with *p<0.05, **p<0.01 and ***p<0.001 vs control (DMSO)

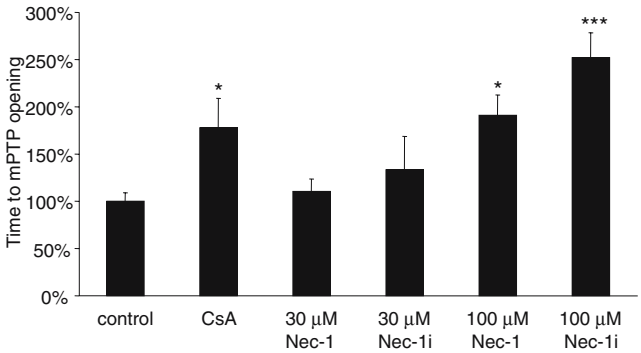

Fig. 3 The times until the initiation of mitochondrial depolarisation i.e. MPTP opening (as assessed using the fluorescent dye tetra-methyl rhodamine methyl ester, TMRM) in the presence and absence of necrostatin-1 (Nec-1; 30 or 100 μM), "inactive" necrostatin-1 (Nec-1i; 30 or 100 μM) or cyclosporin A (CsA; 200 nM). Data are presented as mean±SEM of 3–4 experiments and 19–32 cells per condition with *p<0.05 or ***p<0.001 vs control

Infarct size has been found not to differ significantly between mouse hearts that have been reperfused for 30 and 120 min, indicating that 30 min reperfusion is sufficient to ensure effective washout of dehydrogenase enzymes and cofactors from infarcted tissue, and accurate determination of infarct size [17, 18].

Murine in vivo model of ischemia-reperfusion injury The effects of Nec-1 and Nec-1i on I/R injury were examined in vivo employing a murine model which entailed ligation of the left anterior descending coronary artery (LAD) [19]. C57BL/6J mice (25–35 g) were anaesthetised (ketamine/xylazine/atropine) and the left external jugular vein and right carotid artery cannulated with polyethylene tubing containing heparinised saline (15 U heparin/ml 0.9% saline). The trachea was then cannulated with tubing connected to a ventilator with a tidal volume of 0.2 ml (120 breaths/min). The cannula to the right carotid artery was then clipped and connected to a pressure transducer to monitor mean arterial pressure (MAP). The ECG was monitored via a lead 1 placed subcutaneously in a hind limb. A thoracotomy was then performed between the fourth and fifth ribs and the pericardium carefully retracted to visualise the LAD which was ligated using a 8-0 prolene monofilament polypropylene suture placed approximately 2 mm below the tip of the left auricle. The heart was allowed to stabilise for 15 min prior to ligation for 30 min to induce ischemia. After the ischemic period the ligature was released and a 120 min period of reperfusion followed. In experiments involving Nec-1 or Nec-1i, the compounds were administered as a bolus intra-peritoneal injection

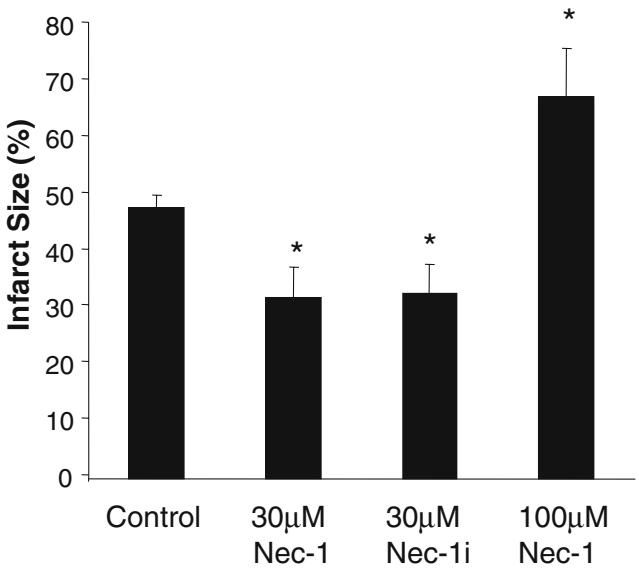

Fig. 4 Reduction of infarct size (expressed as percentage of risk zone, % I/R) in isolated Langendorff perfused mouse hearts with or without necrostatin-1 (Nec-1, 30 or 100 μM) or "inactive" necrostatin-1 (Nec-1i, 30 μM). Values are expressed as mean±SEM (*p<0.05 vs control, n=5–13)

(1.65 mg/kg, Nec-1; 1.74 mg/kg, Nec-1i) so as to coincide with reperfusion: In control experiments 0.2% DMSO was substituted for necrostatin. As for Langendorff experiments TTC was used to determine infarct size the area at risk being established using Evans blue dye.

*Measurement of ROS scavenging ability*Chemiluminescent analysis by emitted light (ABEL®) assays (Knight Scientific UK) incorporating the molluscan photoprotein Pholasin and superoxide and peroxynitrite as substrates were used to investigate the ROS scavenging/antioxidant capacity of Nec-1 (30 and 100 μM).

Statistical analysis Data are given as mean±SEM. Comparisons between more than two groups were made using factorial, one-way analysis of variance (ANOVA). Where a significant F value was obtained the Fisher's protected least significant difference post hoc test was used for between group comparisons. Where only two groups were compared the Student's t test was used. Differences were regarded as statistically significant if a value of p<0.05 was obtained.

Results

Cell death in C2C12 and H9c2 cells Peroxide-induced cell death in cultures of C2C12 cells (which are derived from mouse thigh muscle), as assessed using PI, was reduced markedly in the presence of Nec-1 from 51.2±1.1% (control) to 26.3±2.9% (30 μM Nec-1, p<0.01 vs control) and 17.8±0.9% (100 μM Nec-1, p<0.001), whilst lower concentrations (3 and 10 μM—data not shown) produced smaller, non-significant reductions (Fig. 2a). By contrast, Nec-1i (100 μM) failed to inhibit cell death (50.5±5.5%). With Trolox, a vitamin E analogue with ROS scavenging activity, and our positive control substance, cell death was reduced to 14.3±0.1% (p<0.001 vs control).

In H9c2 cells, which are derived from rat heart, the effects of Nec-1 were less marked, nevertheless, cell death was reduced from 73.0±0.4% (control) to 56.7±0% (30 μM Nec-1, p<0.05 vs control) and 45.4±3.3% (100 μM Nec-1, p<0.01; Fig. 2b). As for C2C12 cells, lower concentrations of Nec-1 (3 and 10 μM) produced only marginal inhibition (data not shown). The antioxidant Trolox diminished cell death to 37.2±1.3% (p<0.001 vs control) and, again, Nec-1i (100 μM) failed to protect cells (66.5±3.4%).

Effect of Nec-1 on MPTP opening in rat cardiomycytes It is generally accepted that cardioprotection, whether induced by preconditioning or postconditioning or by pharmacological means, is linked to activation of the RISK pathway, ultimately leading to delaying of MPTP opening [2, 4]. However, Nec-1 at a concentration of 30 μM failed to influence pore opening (Fig. 3). Similarly, 30 μM Nec-1i also failed to influence mitochondrial depolarisation. By contrast, raising the concentration of Nec-1 to 100 μM increased the time until MPTP opening by 91% (p<0.05 vs control). Nec-1i produced a similar but more marked effect on MPTP opening (+152%, p<0.001 vs control) which, interestingly, exceeded that seen with the positive control

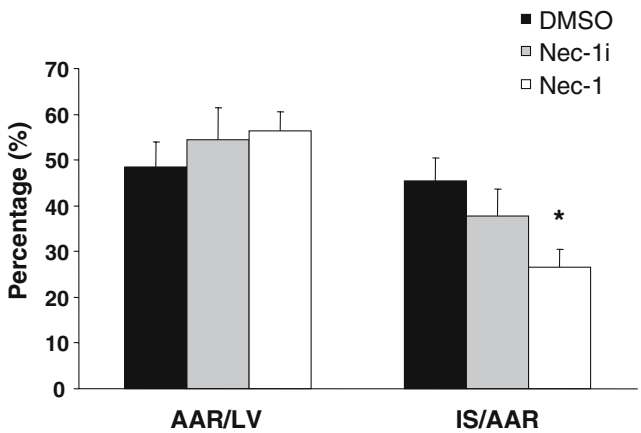

Fig. 5 Reduction in infarct size (IS) in vivo (as a percentage of the area at risk (AAR) in the left ventricle (LV) induced by necrostatin administered during reperfusion (120 min) following regional ischemia precipitated by ligation of the left anterior descending coronary artery (LAD). Necrostatin-1 (Nec-1) or "inactive" necrostatin-1i (Nec-1i) were administered as a bolus injection (Nec-1, 1.65 mg/kg; Nec-1i, 1.74 mg/kg). Data are given as mean±SEM with *p<0.05 (n=6–8)

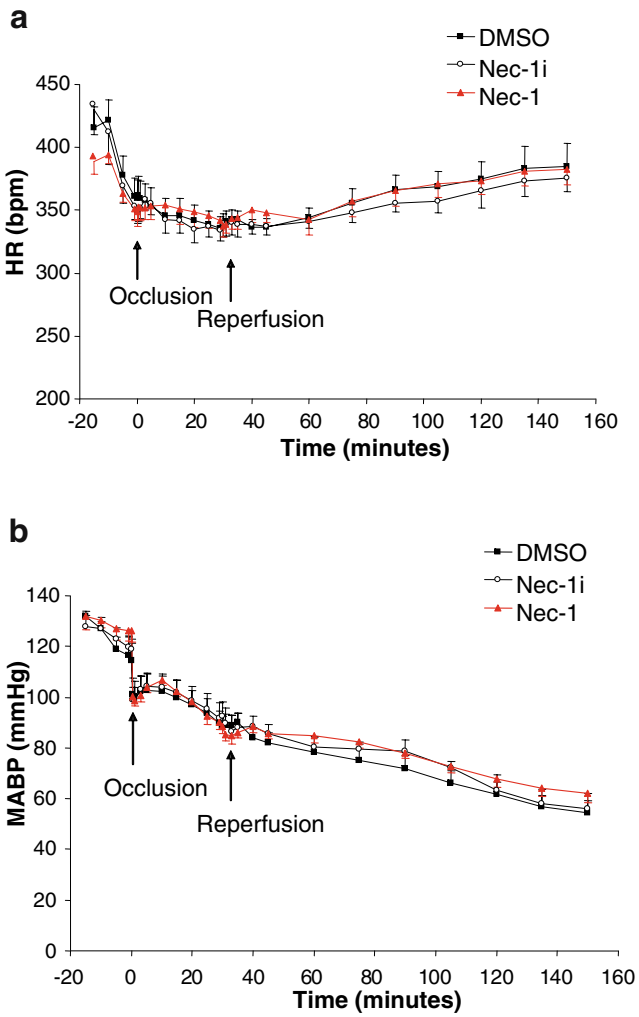

Fig. 6 Heart rate (HR; **a**) and mean arterial blood pressure (MABP; **b**) in the presence and absence of necrostatin-1 (Nec-1, 1.65 mg/kg) or "inactive" necrostatin-1 (Nec-1i, 1.74 mg/kg) administered during reperfusion (120 min) following ischemia (ligation of left anterior descending coronary artery (LAD). Data are presented as mean±SEM of 6–8 experiments

substance Cyclosporin A (CsA; 200 nM; +78%, $p<0.05$), a recognised inhibitor of MPTP opening (Fig. 3).

Effect of Nec-1 on myocardial infarct size in vitro (Langendorff experiments) Experiments carried out using a Langendorff perfused mouse heart model showed that the administration, at reperfusion, of 30 μM Nec-1 reduced infarct size from 48.0±2.0% (control) to 32.1±5.4% ($p<0.05$; Fig. 4). Interestingly, the administration of 100 μM Nec-1 did not result in reduced infarct size, rather, infarct size was increased (48.0±2.0%, control vs 67.1±8.6%, 100 μM Nec-1, $p<0.05$). Perhaps, surprisingly, 30 μM Nec-1i was also found to decrease infarct size (48.0±2.0%, control vs 32.9±5.1%, Nec-1i; $p<0.05$).

Effect of Nec-1 on myocardial infarct size in vivo In anaesthetized C57Bl/6J mice subjected to an I/R protocol, Nec-1 (1.65 mg/kg) administered at reperfusion reduced infarct size from 45.3±5.1% (control) to 26.6±4.0% ($p<0.05$; Fig. 5). Nec-1i (1.74 mg/kg), by contrast, produced only a marginal effect (37.8±6.0%). Neither agent produced any significant hemodynamic changes as evidenced by the data obtained throughout experiments for heart rate (Fig. 6a) and mean arterial pressure (Fig. 6b). The mortality rate for in vivo experiments was 20% and evenly distributed between the control, Nec-1 treated and Nec-1i treated groups.

ROS scavenging ability ABEL® assays, employing superoxide or peroxynitrite as substrates, demonstrated that Nec-1 does not act as an antioxidant. Thus, the rate of superoxide production was not found to be decreased in the presence of 30 μM Nec-1 (15.2±0.7 U/s) or 100 μM Nec-1 (20.0±0.1 U/s) as compared with MeOH control (12.7±0.1 U/s). Similarly, with peroxynitrite as substrate Nec-1 did not exhibit anti-oxidant activity (129.9±1.1 U/s and 159±4.2 U/s, 30 μM Nec-1 and 100 μM Nec-1, respectively vs 101.1±0.1 U/s, control).

Discussion

To summarise our findings, we have shown that peroxide-induced necrosis in C2C12 and H9c2 cells was attenuated by Nec-1. We have also shown that Nec-1 reduced infarct size in both in vivo and in vitro models of I/R injury. In addition, Nec-1 was found to delay the opening of the MPTP, a proposed key determinant of myocardial survival. Our data, therefore, would appear to coincide with that of Degterev et al. [12] as it provides further evidence that Nec-1 is effective in inhibiting cellular damage and reducing I/R injury, albeit in the case of the present study in the heart.

The data obtained with in vivo experiments were comparable to those of Degterev et al. [12], i.e. like Degterev et al. [12] we found that Nec-1 reduced infarct volume whilst Nec-1i produced only marginal effects. By contrast, Langendorff experiments indicated that Nec-1i also reduced infarct size and to extents similar to those achieved with Nec-1. Obviously, differences regarding the data obtained from in vivo and in vitro studies can be explained on the basis that we are dealing with different experimental models that may be under the control of different physiological/biochemical mechanisms. It is, however, also the case that drugs administered in vivo will be subject to pharmacodynamic influences (i.e. metabolism, protein binding etc.) during their transit in the circulation that would not necessarily apply under the conditions occurring in a Langendorff perfusion apparatus.

Another interesting observation concerning the Langendorff studies was that whilst 30 μM Nec-1 protected against

infarction, raising its concentration to 100 μM caused a substantial and significant increase in infarct size. This could indicate that Nec-1 at higher concentrations (and, indeed, concentrations that one would perhaps not expect to see in an in vivo setting) produces non-specific or toxic actions which potentiate apoptotic and necrotic mechanisms, and culminate in enhanced myocardial infarction. Such a finding is not unique and has parallels with data reported previously by this laboratory for other agents. Pioglitazone, for example, was shown to be cardioprotective at lower concentrations but produced adverse effects when its concentrations were increased [6].

When the actions of Nec-1 (and Nec-1i) were examined on MPTP opening some intriguing observations were made. Whilst 30 μM Nec-1 and Nec-1i produced no discernible effects on MPTP opening, raising the concentration of Nec-1 and, especially, Nec-1i to 100 μM increased the times until mitochondrial depolarisation. These findings are of particular interest when considered in the context of the infarct data. As outlined above, 30 μM Nec-1 (and Nec-1i) was found to reduce infarct size whilst increasing its concentration (to 100 μM) appeared to induce myocardial damage. One could speculate that the data obtained with the infarction and MPTP studies with the higher drug concentrations reflect non-specific or toxic effects, whereas, the results yielded with lower necrostatin concentrations relate to specific and beneficial effects that do not involve the MPTP. As delaying MPTP opening is believed to represent a key factor with respect to cardioprotection and may be linked to apoptosis, our failure to show a delay with lower Nec-1 concentrations may be consistent with the findings of Degterev and colleagues [12], who claimed that the effects produced by Nec-1 are mediated by a mechanism distinct from necrosis and apoptosis, namely necroptosis. As suggested by Formigli et al. [20] for a process they termed aponecrosis and claimed shared characteristics with apoptosis and necrosis, necroptosis could represent one of a continuum of death processes with apoptosis and necrosis occurring at its two extremes [20]. Clearly, however, before any firm conclusions can be made regarding necroptosis and the mode(s) of action of necrostatins in the context of myocardial infarction more detailed studies, possibly involving the use of Nec-1 in combination with caspase inhibitors (inhibitors which block apoptotic pathways), should be carried out [21]. An examination of the effects of necrostatins on cell signaling pathways involved in cardioprotection will aid in the characterisation of these agents [4]. The advent of newer and possibly more potent forms of the necrostatins will also assist in this process [22].

In conclusion, as discussed by Degterev et al. [12] necrosis and apoptosis play key roles in a variety of pathological conditions, including myocardial infarction, and the identification of compounds such as the necrostatins which may target the proposed alternative death pathway, necroptosis, may lead to the development of more effective therapies. Our data are the first to indicate that necrostatin is protective against I/R injury in the myocardium, possibly via mechanisms other than those that are generally accepted as being involved (i.e. apoptosis and necrosis) [23]. It should be stressed, however, that these are preliminary studies. Thus, we intend to carry out further, more probing investigations, including in human tissue, (1) to obtain information regarding the therapeutic window for necrostatins, (2) to establish if necroptosis is, indeed, a feature of the degenerative changes occurring in myocardial infarction, (3) to determine the significance of necroptosis in relation to the other forms of cell death that occur in MI, and, (4) to elucidate potential cellular mechanisms underlying such a process in the myocardium.

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