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Inhibitors of the activity of poly (ADP-ribose) synthetase reduce the cell death caused by hydrogen peroxide in human cardiac myoblasts

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1 Poly (ADP-ribose) synthetase (PARS) is a nuclear enzyme activated by strand breaks in DNA which are caused by reactive oxygen species (ROS). Inhibitors of PARS activity reduce the degree of reperfusion injury of the heart *in vivo* and *in vitro*. Here we investigate the role of PARS in the cell death of human cardiac myoblasts caused by hydrogen peroxide.

2 Exposure of human cardiac myoblasts to hydrogen peroxide caused a time- and concentration-dependent reduction in mitochondrial respiration (cell injury), an increase in cell death (LDH release), as well as an increase in PARS activity.

3 The PARS inhibitors 3-aminobenzamide (3 mM), 1,5-dehydroxyisoquinoline (300 μM) or nicotinamide (3 mM) attenuated the cell injury and death as well as the increase in PARS activity caused by hydrogen peroxide (3 mM; 4 h for cell injury/death, 60 min for PARS activity) in human cardiac myoblasts. In contrast, the inactive analogues 3-aminobenzoic acid (3 mM) or nicotinic acid (3 mM) were without effect.

4 The iron chelator deferoxamine (1–10 mM) caused a concentration-dependent reduction in the cell injury and death caused by hydrogen peroxide in these human cardiac myoblasts.

5 Thus, the cell injury/death caused by hydrogen peroxide in human cardiac myoblasts is secondary to the formation of hydroxyl radicals and due to an increase in PARS activity. We therefore propose that activation of PARS contributes to the cell injury/cell death associated with oxidant stress in the heart.

Keywords: Reperfusion injury; PARS inhibitors; oxygen-derived free radicals; myocytes; Girardi cells; oxidant stress

Introduction

There is good evidence that reactive oxygen species (ROS) such as superoxide anions (O_2^-), hydroxyl radicals (OH), and hydrogen peroxide (H_2O_2) as well as peroxynitrite ($ONOO^-$) contribute to reperfusion injury of the previously ischaemic myocardium (Flaherty, 1991; Kukreja & Hess, 1992; Wang & Zweier, 1996). The generation of ROS during either ischaemia or reperfusion has been directly demonstrated using electron paramagnetic resonance spectroscopy (Zweier *et al*., 1987) and chemiluminescence (Henry *et al*., 1988). Interventions which either attenuate the generation or reduce the effects of ROS protect the heart against ischaemia-reperfusion injury. These therapeutic strategies include antioxidant enzymes such as superoxide dismutase and catalase (Jolly *et al*., 1984; Ambrosio *et al*., 1986), radical scavengers such as mannitol and α-tocopherol (Bernier *et al*., 1986), and agents which prevent the generation of radicals such as allopurinol (Manning *et al*., 1984) and deferoxamine (Ambrosio *et al*., 1987). ROS cause cell injury by peroxidation of membrane lipids, denaturation of proteins such as enzymes and ion channels, and DNA injury. For instance, exposure of cultured cells to ROS including H_2O_2, $ONOO^-$ or O_2^- results in single strand breaks in DNA and subsequent activation of the DNA repair enzyme, poly (ADP-ribose) synthetase (PARS) (Lautier *et al*., 1993). Once activated, PARS catalyses the transfer of poly (ADP-ribose) groups from nicotinamide adenine dinucleotide (NAD) to nuclear proteins with concomitant formation of nicotinamide. Under conditions of oxidant stress, DNA injury causes excessive activation of the PARS enzyme resulting in a fall in the intracellular levels of its substrate NAD (Schraufstatter *et al*., 1986b). As NAD is necessary for mitochondrial respiration, depletion of NAD leads to a fall in the intracellular levels of ATP. In addition, the nicotinamide formed when PARS is activated can be recycled to NAD in a reaction that consumes ATP (Carson *et al*., 1986). A decline in the intracellular levels of ATP results in severe cellular dysfunction and ultimately cell death (Schraufstatter *et al*., 1986a). Inhibition of the activity of PARS e.g. with 3-aminobenzamide, prevents the NAD and ATP depletion caused by oxidant stress, and improves cell survival (Schraufstatter *et al*., 1986a). The relative contribution of PARS in mediating cell injury, however, appears to depend on the oxidant used and the cell type studied. There is substantial evidence that activation of PARS contributes to injury of lymphocytes (Schraufstatter *et al*., 1986a), P388D1 cells (a macrophage tumor cell line) (Schraufstatter *et al*., 1986b), endothelial cells (Thies & Autor, 1991), smooth muscle cells (Szabo *et al*., 1996), fibroblasts (Yamamoto *et al*., 1993) and neurones (Zhang *et al*., 1994) exposed to oxidant stress. However, there is no evidence that the activation of PARS contributes to the injury caused by ROS in cardiac cells or cell lines of human origin.

We have recently discovered that the administration, upon reperfusion, of inhibitors of PARS activity attenuate (i) the degree of necrosis caused by regional ischaemia and reperfusion in the anaesthetised rabbit and (ii) attenuate the contractile dysfunction caused by global ischaemia and reperfusion in the isolated, perfused heart of the rabbit (Thiermermann *et al*., 1997). Thus, we have proposed that (i) activation of PARS contributes to reperfusion injury and (ii) that inhibition of PARS activity may be useful in the therapy of reperfusion injury of the heart. The exact mechanism(s) by which PARS inhibitors cause the observed reduction in infarct

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size is not clear. Our results suggest that PARS inhibitors directly protect cardiac myocytes against reperfusion injury, presumably by interfering with the effects of ROS. Alternatively, it has been suggested that inhibitors of PARS activity may reduce infarct size by reducing the infiltration of polymorphonuclear leukocytes into the reperfused myocardium (Zingarelli *et al*., 1997).

This study was designed to elucidate the mechanism by which hydrogen peroxide causes injury of human cardiac myoblasts (Girardi cells). Specifically, we investigate here whether the exposure of human cardiac myoblasts to hydrogen peroxide leads to (i) a fall in mitochondrial respiration and ultimately cell death and (ii) an increase in PARS activity. In addition, we have investigated whether this effect of hydrogen peroxide is secondary to the generation of the highly toxic hydroxyl radical. We have also investigated whether several, chemically distinct inhibitors of PARS activity attenuate the degree of cell injury caused by hydrogen peroxide. Finally, to elucidate whether the 'cardioprotective' effects of PARS inhibitors documented *in vivo* (Thiemermann *et al*., 1997) are due to the ability of these agents to scavenge ROS, we have compared the effects of PARS inhibitors with those of established scavengers of superoxide anions superoxide dismutase (SOD) and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) (Laight *et al*., 1997a).

Methods

Cell culture

Human atrial myoblasts (Girardi cells) were obtained from the European Collection of Cell Cultures (ACACC; Salisbury, Wiltshire, U.K.) and grown to confluence in culture flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (3.5 mM) and 10% foetal calf serum. Cells were passaged every 2 days- removed by treatment with trypsin/EDTA and then cultured in 96-well or 6-well plates (for the measurement of PARS activity only) until they reached confluence. This cell line derives from a biopsy specimen of the right auricular appendage of an adult human heart.

Experimental design

To investigate the effects of exposure of human cardiac myoblasts to hydrogen peroxide, cells were exposed to hydrogen peroxide (10 μM to 10 mM) for various durations (1–6 h) after which cell injury was assessed. To elucidate the effects of PARS inhibitors on the cell injury caused by hydrogen peroxide, cells were preincubated (10 min, 37°C) with (i) the PARS inhibitors (Banasik *et al*., 1992) 3-aminobenzamide (3-AB, 0.1 to 30 mM), 1,5-dihydroxyisoquinoline (ISO, 0.01 to 3 mM) or nicotinamide (Nic, 0.1 to 30 mM), or (ii) their inactive (with respect to inhibition of PARS activity) structural analogues 3-aminobenzoic acid (3-ABA, 0.1 to 3 mM) or nicotinic acid (Nic A, 0.1 to 30 mM) (Banasik *et al*., 1992). The cells were then exposed to hydrogen peroxide (3 mM) for 4 h after which time cell injury/death was assessed.

To elucidate which radical species mediates the cell injury caused by hydrogen peroxide, cells were preincubated with the iron chelator, deferoxamine (0.01 to 10 mM), exposed to hydrogen peroxide (3 mM) for 4 h, after which time cell injury/death was assessed.

To elucidate whether hydrogen peroxide causes PARS activation, cells were exposed to hydrogen peroxide (1–10 mM, concentration-response study) for various durations (10 to 90 min, time-response study). Having found that a maximal increase in PARS activity occurred within 60 min after addition of hydrogen peroxide, the following study was designed to investigate whether the PARS inhibitors used to indeed inhibit PARS activity in human cardiac myoblasts: Cells were (i) preincubated with media containing the PARS inhibitors 3-AB (3 mM), ISO (300 μM) or Nic (3 mM), or their inactive analogues 3-ABA (3 mM) or Nic A (3 mM), (ii) exposed to hydrogen peroxide (3 mM) for 60 min, and (iii) collected and PARS activity was measured as described below.

Measurement of cell injury (MTT assay)

Cell viability was determined indirectly by measuring the mitochondrial-dependent reduction of MTT (3-(4,5-dimethyliazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan (i.e. mitochondrial respiration). Cells in 96-well plates were incubated with MTT (100 μl, 0.2 mg ml^{-1}, dissolved in PBS) for 60 min at 37°C. MTT solution was removed by aspiration and cells were solubilised in 100 μl of dimethyl sulphoxide (DMSO). The amount of purple formazan formed was detected and quantified by measuring the absorbance of the solution at 550 nm using an Anthos Labtec microplate reader (Labtec, Uckfield, Sussex, U.K.). Results are expressed as mitochondrial respiration as a percentage of the control cells (i.e. those not exposed to hydrogen peroxide) which was taken as 100%.

Measurement of cell death (Lactate dehydrogenase assay)

Loss of plasma membrane integrity (cell death) was assessed by measurement of the activity of lactate dehydrogenase (LDH) in the supernatant. A 100 μl sample of cell-free supernatant was transferred into a 96-well plate. LDH activity was measured using a cytotoxicity detection kit (Boehringer Mannheim Ltd, Lewes, Sussex, U.K.). The kit operates on the principle that (in the first step) released LDH reduces NAD to NADH and H^+ by oxidation of lactate to pyruvate. In the second reaction, 2 H are transferred from NADH and H^+ to the yellow tetrazolium salt (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride) by a catalyst. Thus, the increase in amount of enzyme activity in the supernatant directly correlates with the amount of formazan produced. The formazan formed is water soluble and shows a broad absorbance maximum at about 500 nm where the tetrazolium shows no significant absorbance at these wavelengths. So, following addition of 100 μl of kit reaction buffer to the sample of cell supernatant, the plates were incubated at room temperature in the dark for 30 min. The reaction was terminated by addition of 25 μl 2 M HCl and amount of formazan formed was detected and quantified by measuring the absorbance of the solution at 490 nm (reference filter 620 nm) using a Ceres microplate reader (Bio-Tek Instruments Inc., Winooski, V.T., U.S.A.). Results are expressed as percentage of the total LDH activity released by Triton-X (1%).

Measurement of PARS activity in human cardiac myoblasts

PARS activity was measured as the ability of permeabilised cells to transfer the substrate [^{3}H]-NAD onto nuclear proteins over a set time period as described by Schraufstatter *et al*., 1986b. Following the appropriate treatment and duration, the media was aspirated before addition of fresh culture medium (400 μl) and the cells were scraped and transferred to an

Eppendorff tube. Following centrifugation (10,000 *g*, 10 s) and aspiration of media, the cells were resuspended in reaction buffer (56 mM HEPES buffer containing 28 mM potassium chloride, 28 mM sodium chloride, 2 mM magnesium chloride, 0.02% digitonin, and 125 nmoles NAD spiked with 0.25 μCi/ml [^{3}H-NAD], pH 7.5), vortexed for 5 s and incubated at 37°C for 5 min. The reaction was terminated by addition of 200 μl of 50% trichloroacetic acid (TCA) and the resultant precipitate was pelleted by centrifugation at 10,000 *g* for 3 min. The protein pellet was washed twice with 50% TCA and then solubilised in 200 μl 1 M NaOH/2% SDS overnight at 37°C in a shaking incubator (Luckham, Basingstoke, Hampshire, U.K.). The radioactivity incorporated into protein was determined by scintillation counting (Beckman Instruments Ltd, High Wycombe, Buckinghamshire, U.K.).

Evaluation of the effects of test drugs on the reduction of cytochrome c caused by superoxide anion generation (by hypoxanthine/xanthine oxidase)

The ability of the test compounds to scavenge superoxide anions was determined in an *in vitro* microassay as described by Laight *et al.* (1997b). The assay mixture consisted of (final concentration): 50 μl ferricytochrome c (cyt c, 100 μM), 10 μl xanthine oxidase (XO, 10 mU ml^{-1}), 20 μl hypoxanthine (Hx, 100 μM) and 10 μl catalase (200 U ml^{-1}) dissolved in phosphate buffered saline (10 mM phosphate, pH = 7.4), and 10 μl of the test compounds. Changes in absorbance (A) at 550 nm were recorded at 37°C using a kinetic plate reader (Anthos Labtec, Uckfield, Sussex, U.K.) over 3 min. Using this technique we have compared the effects the PARS inhibitors 3-AB (0.1 to 3 mM), ISO 0.01 to 3 mM) and Nic (0.1 to 3 mM) with those of the established superoxide anion scavengers superoxide dismutase (SOD, 200 U ml^{-1}) or 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO, 1 mM) (Laight *et al.*, 1997a).

Statistical analysis

All data are expressed as mean ± s.e.mean of *n* independent experiments. Statistical comparisons between groups were made by a one way ANOVA followed by a Dunnett's test. A *P* value of less than 0.05 was considered to be statistically significant.

Drugs and materials

Unless otherwise stated all compounds were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Dulbecco's modified Eagle's medium (DMEM) was from Life Technology Ltd (Paisley, Lanarkshire, U.K.). 1,5-dihydroxyisoquinoline was obtained from Aldrich (Poole, Dorset, U.K.) and dissolved in 10% DMSO. [^{3}H]-NAD was obtained from NEN Life Science Products (Hounslow, Middlesex, U.K.).

Results

Inhibitors of PARS activity attenuate the impairment in mitochondrial respiration and the cell death caused by hydrogen peroxide in human cardiac myoblasts

In human cardiac myoblasts, hydrogen peroxide caused a concentration- and time-dependent fall in mitochondrial respiration (Figure 1). Hydrogen peroxide also caused a time- and concentration-dependent release into the culture medium of LDH (Figure 2). Inhibitors of PARS activity including 3-aminobenzamide (3-AB, 0.1 to 30 mM), 1,5-dihydroxyisoquinoline (ISO, 0.01 to 3 mM) and nicotinamide (Nic, 0.1 to 30 mM) caused a concentration-dependent attenuation of the impairment in mitochondrial respiration caused by hydrogen peroxide (3 mM for 4 h) (Figure 3). When compared to 3-AB (3 mM), 3-aminobenzoic acid (3-ABA, 3 mM), the structure of which is very similar to 3-AB, did not have any effect (Figure 4). Similarly, when compared to nicotinamide (3 mM) nicotinic acid (3 mM) which has a similar structure to Nic, did not affect the impairment in mitochondrial respiration caused by hydrogen peroxide (Figure 4). The PARS inhibitors 3-AB (3 mM), ISO (300 μM) or Nic (3 mM) also attenuated the release of LDH caused by exposure of human cardiac myoblasts to hydrogen peroxide (3 mM for 4 h) where the structural analogues (negative controls) 3-ABA (3 mM) or NicA (3 mM) did not affect the increase in release of LDH caused by hydrogen peroxide (Figure 5).

The cell injury caused by hydrogen peroxide is mediated by generation of the hydroxyl radical in human cardiac myoblasts

The intracellular iron-chelator deferoxamine, which prevents the formation of hydroxyl radicals from hydrogen peroxide (by inhibition of the Fenton reaction), attenuated the fall in mitochondrial respiration (Figure 6) and the associated release of LDH (Figure 7) caused by exposure of human cardiac myoblasts to hydrogen peroxide.

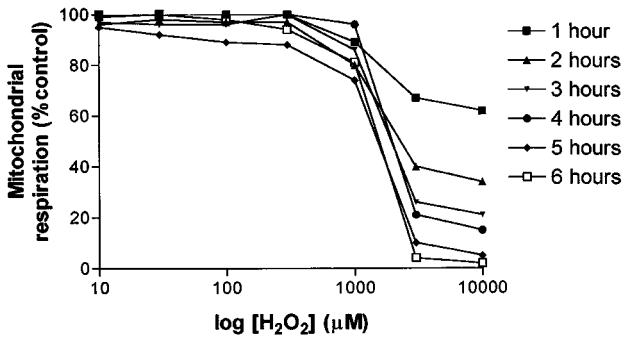

Figure 1 Exposure of human cardiac myoblasts to hydrogen peroxide causes a time- and concentration-dependent reduction in mitochondrial respiration. Data are expressed as mean ± s.e.mean of six observations.

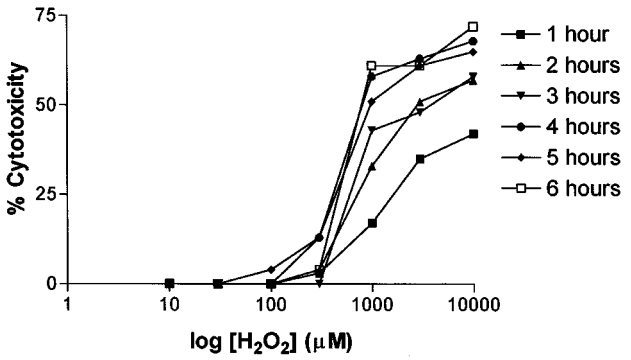

Figure 2 Exposure of human cardiac myoblasts to hydrogen peroxide causes a time- and concentration-dependent increase in the release of lactate dehydrodenase, an indicator of cell death, into the cell supernatant. Data are expressed as mean ± s.e.mean of six observations.

Inhibitors of PARS activity attenuate the increase in PARS activity caused by hydrogen peroxide in human cardiac myoblasts

Exposure of human cardiac myoblasts to hydrogen peroxide (1–10 mM, 10–90 min) caused a time- and concentration-dependent increase in PARS activity (Figure 8a, b). Exposure of the human cardiac myoblasts to hydrogen peroxide (3 mM) for 60 min caused a peak increase in PARS activity. This increase in PARS activity (3 mM, 60 min) was attenuated by pretreatment of the cells with the PARS inhibitors 3-AB (3 mM), ISO (300 μM) or Nic (3 mM) (Figure 8c). In contrast, 3-aminobenzoic acid or nicotinic acid (negative controls) did not affect the increase in PARS activity caused by hydrogen peroxide in human cardiac myoblasts (Figure 8c).

PARS inhibitors do not scavenge superoxide anions in vitro

TEMPO (1 mM) abolished and SOD (200 U ml^{-1}) significantly depressed the rate of reduction of cytochrome c by the

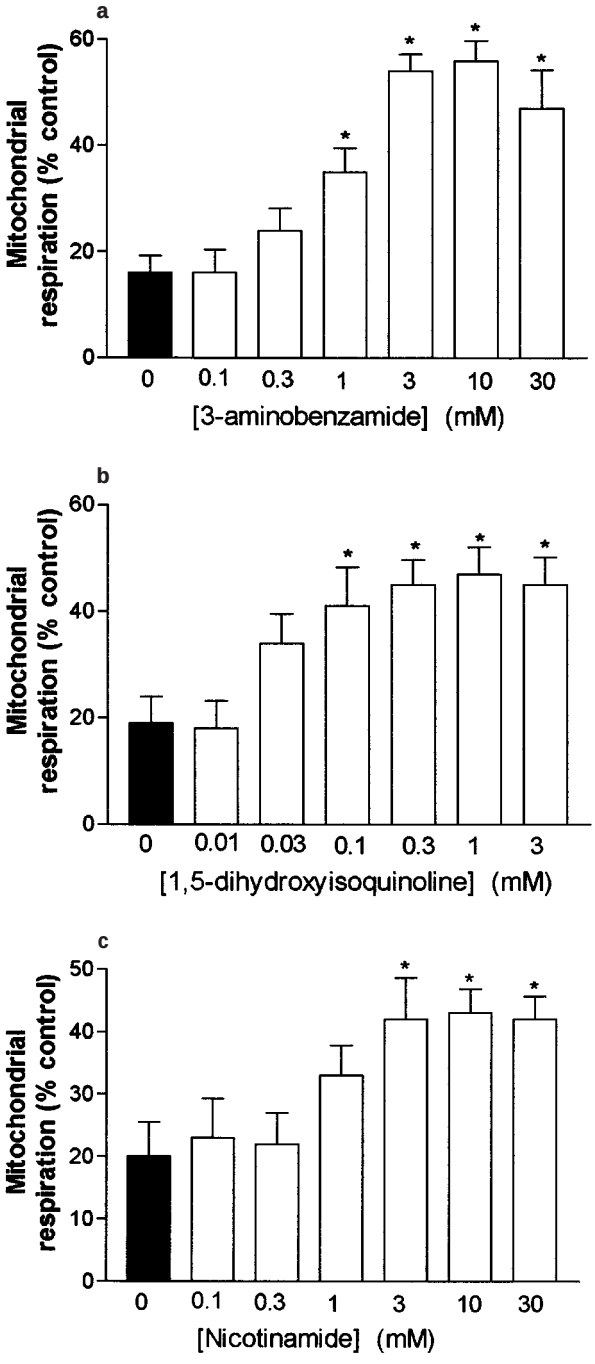

Figure 3 The effect of various chemically distinct inhibitors of PARS activity on the impairment in mitochondrial respiration caused by hydrogen peroxide (H_2O_2, 3 mM, $n=4$) in human cardiac myoblasts. The PARS inhibitors (a) 3-aminobenzamide (3-AB, 0.1 to 30 mM, $n=4$), (b) 1,5-dihydroxyisoquinoline (ISO, 0.01 to 3 mM, $n=4$) or (c) nicotinamide (Nic, 0.1 to 3 mM, $n=4$) cause a concentration-dependent attenuation of the impairment in mitochondrial respiration caused by H_2O_2. Data are expressed as mean$\pm$s.e.mean of n observations $^*P<0.05$ when compared with H_2O_2-control (solid column).

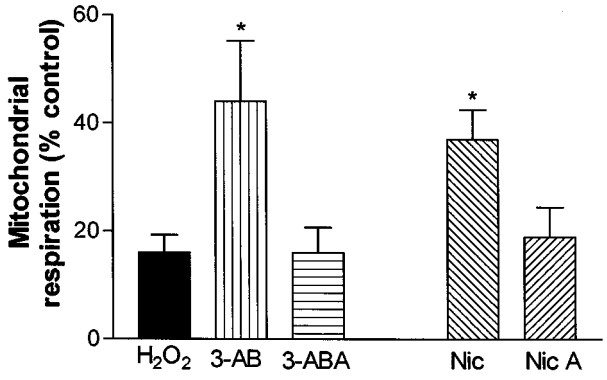

Figure 4 The effect of the inhibitors of PARS activity and their structural analogues on the impairment in mitochondrial respiration caused by hydrogen peroxide (H_2O_2, 3 mM, $n=6$) in human cardiac myoblasts. When compared to 3-AB (3 mM, $n=4$), 3-aminobenzoic acid (3-ABA, 3 mM, $n=4$), the structure of which is very similar to 3-AB, does not have any effect. Similarly, when compared to nicotinamide (3 mM, $n=4$) nicotinic acid (3 mM, $n=4$) which has a similar structure to Nic, does not affect the impairment in mitochondrial respiration caused by hydrogen peroxide. Data are expressed as mean$\pm$s.e.mean of n observations $^*P<0.05$ when compared with H_2O_2-control.

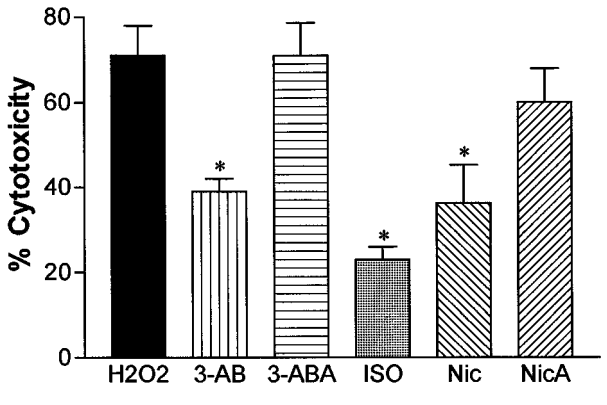

Figure 5 The effect of various chemically distinct inhibitors of PARS activity on the cell death (measured as release of LDH into the cell supernatant) caused by hydrogen peroxide (H_2O_2, 3 mM, 4 h, $n=4$) in human cardiac myoblasts. The PARS inhibitors 3-aminobenzamide (3-AB, 3 mM, $n=4$), 1,5-dihydroxyisoquinoline (ISO, 300 μM, $n=4$) or nicotinamide (Nic, 3 mM, $n=4$) attenuate the increase in cell death caused by H_2O_2. The structural analogues 3-aminobenzoic acid (3-ABA, 3 mM, $n=4$) and nicotinic acid (NicA, 3 mM, $n=4$) do not have any effect. Data are expressed as mean$\pm$s.e.mean of n observations $^*P<0.05$ when compared with H_2O_2-control.

XO/Hx system. In contrast, none of the PARS inhibitors, even at the highest concentrations (Figure 9) had any significant effect on the rate of reduction of cytochrome c.

Discussion

In human cardiac myoblasts, hydrogen peroxide caused a time- and concentration-dependent fall in mitochondrial respiration and subsequently the release of the cytosolic enzyme LDH into the supernatant. Thus, hydrogen peroxide caused within 4 h, an impairment in mitochondrial function and subsequently the death of the human cardiac myoblasts. The cell injury caused by hydrogen peroxide is secondary to the generation of hydroxyl radical, as it was attenuated by the iron chelator deferoxamine (this study), which prevents the formation of hydroxyl radical from hydrogen peroxide by inhibition of the Fenton-reaction. There is good evidence that (i) oxygen-derived free radicals cause strand breaks in DNA in many cell types including rat cardiac myoblasts (Okamato *et al*., 1996), and (ii) that strand breaks in DNA activate PARS (Schraufstatter *et al*., 1986a). Thus, we have hypothesised that the activation of this enzyme may contribute to the injury associated with oxidant stress in cardiac cells. We report here that pretreatment of human cardiac myoblasts with three, chemically distinct PARS inhibitors (3-aminobenzamide, 1,5-dihydroxyisoquinoline or nicotinamide) attenuates both the impairment in mitochondrial respiration as well as the cell death caused by hydrogen peroxide. We, therefore propose that the activation of PARS

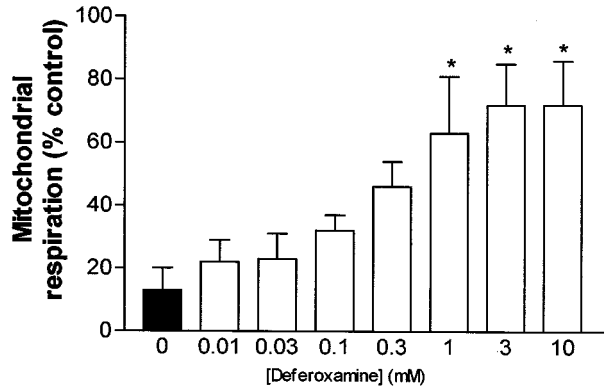

Figure 6 The effect of the iron chelator, deferoxamine on the impairment in mitochondrial respiration caused by hydrogen peroxide (H_2O_2, 3 mM, $n=4$) in human cardiac myoblasts. Deferoxamine causes a concentration-dependent attenuation of the impairment in mitochondrial respiration caused by H_2O_2. Data are expressed as mean$\pm$s.e.mean of n observations $^*P<0.05$ when compared with H_2O_2-control.

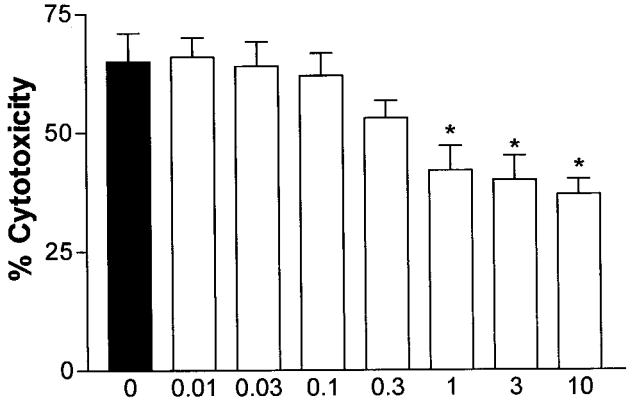

Figure 7 The effect of the iron chelator, deferoxamine on the cell death (measured as release of LDH into the cell supernatant) caused by hydrogen peroxide (H_2O_2, 3 mM, 4 h, $n=4$) in human cardiac myoblasts. Deferoxamine causes a concentration-dependent attenuation of the cell death caused by H_2O_2. Data are expressed as mean$\pm$s.e.mean of n observations $^*P<0.05$ when compared with H_2O_2-control.

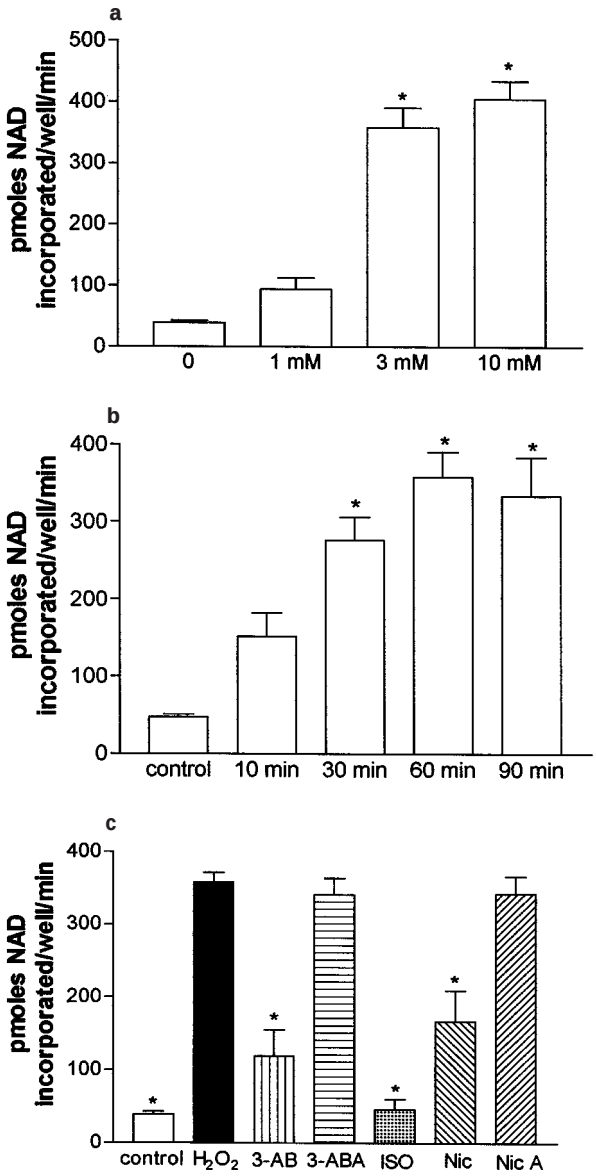

Figure 8 The effect of various chemically distinct inhibitors of PARS activity on the increase in PARS activity caused by hydrogen peroxide: (a) hydrogen peroxide (1–10 mM, $n=6$) causes a concentration-dependent increase in PARS activity (measured at 60 min). (b) hydrogen peroxide (3 mM) causes a time-dependent increase in PARS activity ($n=6$). (c) The PARS inhibitors 3-aminobenzamide (3-AB, 3 mM, $n=6$), 1,5-dehydroxyisoquinoline (ISO, 300 μM, $n=6$) or nicotinamide (Nic, 3 mM, $n=6$) attenuate the increase in PARS activity caused by H_2O_2. 3-aminobenzoic acid (3-ABA, 3 mM, $n=6$) or nicotinic acid (NicA, 3 mM, $n=6$) do not have any effect. Data are expressed as mean$\pm$s.e.mean of n observations $^*P<0.05$ when compared with control (Figures 8a and b) or when compared to H_2O_2 (Figure 8c).

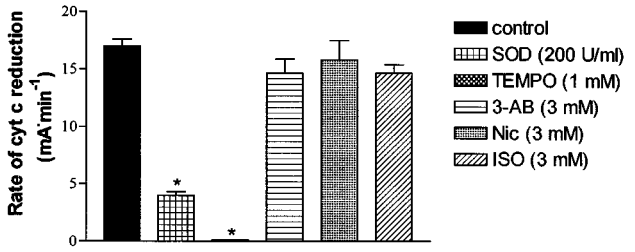

Figure 9 Effect of inhibitors of PARS activity on the rate of cytochrome c reduction by a xanthine oxidase/hypoxanthine system. TEMPO (1 mM, $n=4$) or superoxide dismutase (SOD, 200 U ml^{-1}) reduced the rate of cyt c reduction indicating superoxide anion scavenging activity. The PARS inhibitors 3-aminobenzamide (3-AB, 3 mM), 1,5-dihydroxyisoquinoline (ISO, 300 μM, $n=4$) or nicotinamide (Nic, 3 mM, $n=4$) did not have any effect. Data are expressed as mean$\pm$s.e.mean of n observations $^*P<0.05$ when compared with control ($n=4$).

contributes to the cell injury/death caused by hydrogen peroxide in human cardiac myoblasts. This hypothesis is supported by the following, four key findings: (1) Exposure of human cardiomyoblasts to hydrogen peroxide caused within 60 min a substantial increase in PARS activity. (2) This increase in PARS activity was attenuated by all of the PARS inhibitors employed. (3) At concentrations which abolished the increase in PARS activity, all of the PARS inhibitors used caused a significant reduction in the impairment in mitochondrial function and cell death caused by hydrogen peroxide. (4) 3-aminobenzoic acid, the structure of which is very similar to 3-AB, did neither inhibit the increase in PARS activity nor the cell injury/death caused by hydrogen peroxide (negative control).

Our conclusion that the activation of PARS contributes to the cell injury/death caused by hydrogen peroxide in human cardiac myoblasts is also supported by the following arguments: One could argue that the beneficial effects of 3-AB are due to effects which are not related to the ability of this benzamide to inhibit PARS activity (non-specific effects). This is, however, not the case, as the structural analogue of 3-AB, namely 3-ABA, did not reduce the cell injury/death caused by hydrogen peroxide in human cardiac myoblasts. On the contrary, our finding that 3-ABA did neither inhibit PARS activity nor the cell injury caused by hydrogen peroxide strongly supports our hypothesis that the inhibition of PARS activity leads to the protection of human cardiac myoblasts against oxidant stress.

One could also argue that the beneficial effects of nicotinamide observed in our study may be due to an enhancement of the intracellular levels of NAD (as nicotinamide can be re-synthesized to NAD, Carson *et al*., 1986). This is, however, not the case, as nicotinic acid, which can also be re-synthesized to NAD, did not reduce the degree of cell injury/death caused by hydrogen peroxide. Most notably, nicotinic acid did also not inhibit the increase in PARS activity caused by hydrogen peroxide in human cardiac myoblasts.

It should be noted that the reduction of either cell injury or death afforded by the PARS inhibitors used in this study or in the *in vivo* setting of myocardial ischaemia-reperfusion injury (Thiemermann *et al*., 1997) is unlikely to be due to the ability of these agents to scavenge oxygen-derived free radicals, as none of the PARS inhibitors used (at concentrations which inhibited PARS activity and prevented cell injury/death) scavenged superoxide anions.

We have recently discovered that the PARS inhibitors used in this study reduce the degree of infarction caused by ischaemia and reperfusion of the heart and skeletal muscle in the rabbit (Thiemermann *et al*., 1997). Our conclusions (Thiemermann *et al*., 1997) that (i) the activation of PARS contributes to ischaemia-reperfusion injury and (ii) that inhibitors of PARS activity may be useful in the therapy of ischaemia-reperfusion injury of the heart and other organs has recently been supported by the following publications: (1) Inhibition of PARS activity reduces the infarct size arising from ischaemia reperfusion of the rat heart (Zingarelli *et al*., 1997), mouse brain (Eliasson *et al*., 1997; Endres *et al*., 1997) and rat gut (Cuzzocrea *et al*., 1997). (2) The cerebral infarct size is significantly smaller in mice in which the PARS gene has been deleted by gene-targeting (Eliasson *et al*., 1997; Endres *et al*., 1997). The mechanism by which inhibitors of PARS activity reduce infarct size in the heart and other organs is largely unclear. It has been suggested that PARS inhibitors like 3-AB enhance the 'rate of detachment' of neutrophils from the endothelium (Szabo *et al*., 1997) suggesting that these agents attenuate reperfusion injury by reducing the infiltration of activated neutrophils. Clearly, our study demonstrates that inhibitors of PARS activity can protect human cardiomyoblasts against hydrogen peroxide suggesting that these agents can directly protect cardiac myocytes. This notion is also supported by our finding that 3-AB reduces the infarct size caused by ischaemia-reperfusion of the isolated, buffer-perfused heart of the rabbit (Thiemermanan *et al*., 1997). Thus, further studies are necessary to elucidate if, and to what extent, the reduction of neutrophil extravasation and/or protection of cardiac myocytes contributes to the cardioprotective effects of PARS inhibitors.

In conclusion, this study demonstrates that the cell injury/death caused by hydrogen peroxide in human cardiac myoblasts is (i) secondary to the formation of hydroxyl radical, (ii) due to an increase in PARS activity and (iii) attenuated by inhibitors of PARS activity. The results suggest that the cardioprotective mechanism of action of PARS inhibitors *in vivo* are (at least in part) due to a direct protection of cardiac myocytes against reperfusion injury, by interfering with the downstream events following exposure to ROS. We propose that these inhibitors of PARS activity may be useful in the therapy of other disorders of the heart which are associated with oxidant stress.

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