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Reduction of myocardial reperfusion injury by an inhibitor of poly (ADP-ribose) synthetase in the pig

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Abstract

The effect of the Poly (adenosine 5′-diphosphate ribose) synthetase (PARS) inhibitor 3-aminobenzamide on (i) infarct size caused by regional myocardial ischaemia (60 min) and reperfusion (3 h) in the anaesthetised pig, and (ii) on the cell injury/necrosis of human cardiomyoblasts caused by hydrogen peroxide (3 mM) was investigated. Regional myocardial ischaemia and reperfusion resulted in an infarct size of 66 ± 3% of the area at risk, which was reduced by 3-aminobenzamide (to 44 ± 2%, $n = 6$), but not 3-aminobenzoic acid (66 ± 5%, $n = 4$). 3-aminobenzamide also reduced the postischaemic contractile dysfunction. 3-aminobenzamide, but not 3-aminobenzoic acid, abolished the increase in PARS activity as well as the cell injury/necrosis caused by hydrogen peroxide in the cardiomyoblasts. In conclusion, the PARS inhibitor 3-aminobenzamide reduces myocardial reperfusion injury in the pig, and attenuates the cell injury and death associated with oxidant stress in human cardiomyoblasts. We propose that the activation of PARS plays an important role in the injury associated with oxidant stress of the heart.

Keywords: PARS (Poly (adenosine 5′-diphosphate ribose) synthetase); Heart; Reperfusion injury; Oxygen radical; Cardiomyoblast, human; (Pig)

1. Introduction

Poly (adenosine 5′-diphosphate ribose) synthetase (PARS; E.C. 2.4.2.30) is a chromatin-bound enzyme which is constitutively expressed in most eukaryotic cells (Lautier et al., 1993). The physiological role of this enzyme is still unclear, but there is substantial evidence that it plays a role in the repair of strand breaks in DNA (Satoh and Lindahl, 1992). Exposure of cultured cells to reactive oxygen species including hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$), peroxynitrite ($ONOO^-$) or superoxide anions (O_2^-) results in single strand breaks in DNA and subsequent activation of PARS (Schraufstatter et al., 1986a; Szabo et al., 1996). PARS catalyses the transfer of ADP-ribose groups from nicotinamide adenine dinucleotide (NAD) to nuclear proteins with concomitant formation of poly (ADP-ribose) and 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., 1986a). As NAD is necessary for the synthesis of ATP, 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., 1986b). Inhibitors of PARS activity attenuate the fall in NAD and ATP and improve survival of cultured cells (e.g., fibroblasts (Yamamoto et al., 1993), endothelial cells (Thies and Autor, 1991), neurones (Zhang et al., 1994), smooth muscle cells (Szabo et al., 1996)) exposed to oxygen-derived free radicals or peroxynitrite.

Reactive oxygen species such as O_2^-, $OH^{\cdot}$ and H_2O_2 as well as $ONOO^-$ contribute to reperfusion injury of the previously ischaemic myocardium (Flaherty, 1991; Kukreja and Hess, 1992; Wang and Zweier, 1996). The generation of reactive oxygen species during either ischaemia or reperfusion has been directly demonstrated using electron

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paramagnetic resonance spectroscopy (Zweier et al., 1986) and chemiluminescence (Henry et al., 1988). Interventions which either attenuate the generation or reduce the effects of reactive oxygen species protect the heart against ischaemia-reperfusion injury (Jolly et al., 1984; Ambrosio et al., 1986; Bernier et al., 1986; Ambrosio et al., 1987).

We have recently discovered that the administration, just prior to reperfusion, of various chemically distinct inhibitors of PARS activity attenuate the degree of necrosis caused by regional myocardial ischaemia and reperfusion in the anaesthetised rabbit. In addition, reperfusion of hearts with buffer containing the PARS inhibitor 3-aminobenzamide attenuates the contractile dysfunction caused by global ischaemia and reperfusion in the isolated, perfused heart of the rabbit (Thiemermann et al., 1997). Thus, we have proposed that (i) activation of PARS contributes to reperfusion injury and (ii) inhibition of PARS activity may be useful in the therapy of reperfusion injury of the heart (Thiemermann et al., 1997). This proposal is supported by the recent finding that 3-aminobenzamide also reduces the infarct size caused by regional myocardial ischaemia and reperfusion of the rat heart in vivo and in vitro (Zingarelli et al., 1997; Bowes et al., 1998). It is unknown whether inhibitors of PARS activity (i) can protect the heart of higher species against reperfusion injury, and (ii) protect human cardiac cells against oxidant stress.

Thus, this study was designed to evaluate the effect of the PARS inhibitor 3-aminobenzamide on infarct size caused by regional myocardial ischaemia and reperfusion in the anaesthetised, open-chest pig. In addition, we have evaluated whether administration, upon reperfusion, of this PARS inhibitor affects the contractile dysfunction associated with regional myocardial ischaemia-reperfusion in this species. Finally, we have also investigated whether (i) hydrogen peroxide can increase PARS activity in human cardiomyoblasts, and (ii) 3-aminobenzamide prevents the cell injury and necrosis caused by hydrogen peroxide in these cells.

2. Methods

2.1. Surgical procedure

Twenty four pigs weighing between 23–30 kg were sedated with Ketamin®/Xylazin® (40 mg/kg and 0.8 mg/kg i.m., respectively) and subsequently anaesthetised with sodium pentobarbital (25 mg/kg i.v. as a bolus followed by an i.v. infusion at a rate of 20 mg/kg per h) and ventilated with a Bird-Mark-8 respirator delivering room air (ventilation rate: 16 strokes/min; tidal volume: 400–500 ml). Oxygen was supplied via the respirator as required (1–2 l/min) in order to maintain arterial blood pO_2 values at approximately 100 mmHg and pCO_2 values at approximately 35 mmHg. A thoracotomy was performed through the fifth intercostal space, the lungs were retracted and the heart suspended in a pericardial cradle. The left descending coronary artery was freed from adjacent tissue below the first diagonal branch and a suture was placed around the artery for the later occlusion. The systemic mean arterial pressure was measured with a catheter-tip manometer (Type SPC 350 MR Millar Instruments, Houston, TX, USA) placed in a femoral artery and the left ventricular pressure was recorded with another tip-catheter which had been passed retrogradely from a carotid artery into the left ventricle. Heart rate was calculated from the left ventricular pressure waveform and myocardial contractility (dp/dt_{max}) was measured as the rate of rise of left ventricular pressure. Haemodynamic data were recorded continuously during the course of the experiment on a MK 260 Brush Gould (Cleveland, OH, USA) polygraph.

2.2. Experimental protocol

Following a 60 min equilibration period, the coronary artery was occluded with a snare for 60 min to induce myocardial ischaemia. Reperfusion was accomplished by releasing the snare placed around the coronary artery. The duration of reperfusion was 180 min. The PARS inhibitor 3-aminobenzamide (10 mg/kg) or its inactive analogue 3-aminobenzoic acid (10 mg/kg) (Banasik et al., 1992) were injected into the left ventricle 1 min prior to reperfusion. This dose has previously been shown to reduce infarct size caused by myocardial ischaemia and reperfusion in the anaesthetised rabbit (Thiemermann et al., 1997). At the end of the reperfusion period, animals were killed with an overdose of anaesthetic and the heart removed for assessment of infarct size.

2.3. Determination of infarct size

The heart was excised, the aorta cannulated and the heart perfused retrogradely with two solutions at a constant pressure of 100 mmHg (37°C) at the same time. Evans blue (0.5%) was infused to determine the anatomic area at risk (nonperfused myocardium) and the nonischaemic area. Subsequently, 2,3,5-triphenyltetrazolium chloride (1.5%, dissolved in a 20 mM phosphate buffer, pH 7.4) was infused into the coronary artery just distal to the site of occlusion. In the presence of intact dehydrogenase enzyme systems (normal myocardium), 2,3,5-triphenyltetrazolium chloride forms a brick-red colour, whilst areas of necrosis lack dehydrogenase activity and therefore do not stain. Thus, the viable areas located within the area at risk were stained red, while the necrotic myocardium (within the area at risk) remained unstained (pale yellowish colour) if infarcted. The right ventricle was removed and the left ventricle plus septum was cut into slices, approximately 1.0 cm thick, from the base of the apex. Each slice was placed in a petri dish filled with saline and photographed. The area at risk and the infarcted area (in each slice) were

determined from the relevant colour photographs using computer-assisted planimetry (computer software Elas, Leitz, Wetzlar, Germany). The area at risk was calculated as percentage of the (left ventricle + septum) and the infarcted area as percentage of the area at risk.

2.4. Culture of human cardiomyoblasts

Human atrial myoblasts (Girardi cells) were obtained from the European Collection of cell cultures (ACACC; Salisbury, UK) 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.

2.5. Experimental design (in vitro studies)

To investigate the effects of exposure of human cardiomyoblasts to hydrogen peroxide, cells were exposed to hydrogen peroxide (10 μM to 10 mM) for 4 h and cell injury/necrosis 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 the PARS inhibitor 3-aminobenzamide (3 mM) or its inactive (with respect to inhibition of PARS activity) structural analogue, 3-aminobenzoic acid (3 mM) (Banasik et al., 1992). The cells were then exposed to hydrogen peroxide (3 mM) for 4 h after which time cell injury/necrosis was assessed (see below). To elucidate whether hydrogen peroxide causes PARS activation, cells were exposed to hydrogen peroxide (3 mM) for 10–90 min. Having found that this protocol results in a maximal increase in PARS activity at 60 min, the following study was designed to investigate whether the PARS inhibitors used do indeed inhibit PARS activity in human cardiomyoblasts: Cells were preincubated with media containing the PARS inhibitors 3-aminobenzamide (3 mM) or 3-aminobenzoic acid (3 mM) and then exposed to hydrogen peroxide (3 mM) for 60 min. Thereafter, the cells were collected and PARS activity was measured as described below. In this set of experiments, the cells were treated with the PARS inhibitor whilst they had intact cell membranes. Thus, the degree of inhibition of PARS activity observed is influenced by (i) the ability of the compound to cross the cell membrane, and diffuse to the nucleus and (ii) its potency as an inhibitor of PARS activity (iii) any effects the compound may have on the upstream events, i.e., DNA damage caused by hydrogen peroxide. To eliminate these factors, we investigated the effects of 3-aminobenzamide (or 3-aminobenzoic acid) on the increase in PARS activity in permeabilised cells. Cells were exposed to hydrogen peroxide (3 mM for 60 min to cause a maximal increase in PARS activity) and then permeabilised in reaction buffer (see below) containing 3-aminobenzamide (3 mM) or 3-aminobenzoic acid (3 mM). Thus, in this set of experiments the PARS inhibitor could directly access the active enzyme. This enables the investigator to ensure that the test compound is exerting its effect by inhibition of PARS activity.

2.6. Measurement of cell injury (MTT assay) and cell necrosis (LDH 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, dissolved in PBS) for 60 min at 37°C. MTT solution was removed by aspiration and cells were solubilised in 100 μl of dimethyl sulfoxide (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.

Loss of plasma membrane integrity (cell necrosis) 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, Lewes, UK). 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, two 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.

2.7. Measurement of PARS activity in human cardiomyoblasts

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 previously described (Schraufstatter et al., 1986a). 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 nmol NAD spiked with 0.5 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% trichloroacteic acid and the resultant precipitate was pelleted by centrifugation at 10 000 × *g* for 3 min. The protein pellet was washed twice with 50% trichloroacteic acid and then solubilised in 200 μl 1 M NaOH/2% SDS overnight at 37°C in a shaking incubator. The radioactivity incorporated into protein was determined by scintillation counting.

2.8. Materials

Unless otherwise stated, all compounds were purchased from Sigma (Poole, UK). The lactate dehydrogenase assay kit was obtained from Boehringer Mannheim. Dulbecco's modified Eagle's medium (DMEM) was from Life Technology (Paisley, UK). 1,5-dihydroxyisoquinoline was obtained from Aldrich (Poole, UK) and dissolved in 10% DMSO. [^{3}H]NAD was obtained from NEN Life Science Products (Hounslow, UK).

2.9. Statistical analysis

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

3. Results

Of the 24 pigs which underwent coronary artery occlusion, eight died during the course of the experiment (five controls, one treated with 3-aminobenzamide and two treated with 3-aminobenzoic acid) due to ventricular fibrillation. The data obtained from these animals were excluded from data analysis.

3.1. Area at risk and infarct size

The area at risk ranged from 39 to 43% of the left ventricle (including the septum) and was not statistically significantly different between any of the experimental groups studied (Fig. 1a). In control pigs, coronary artery occlusion (60 min) and reperfusion (3 h) resulted in an infarct size of 66 ± 3% of the area at risk ($n = 6$; Fig. 1b). Injection upon reperfusion of the PARS inhibitor 3-aminobenzamide (10 mg/kg) caused a significant (~ 30%) reduction in infarct size when compared to the control group ($n = 6$; Fig. 1b). In contrast, the inactive structural analogue of this PARS inhibitor, 3-aminobenzoic acid, did not have any effect infarct size (when compared to control, $n = 4$; Fig. 1b).

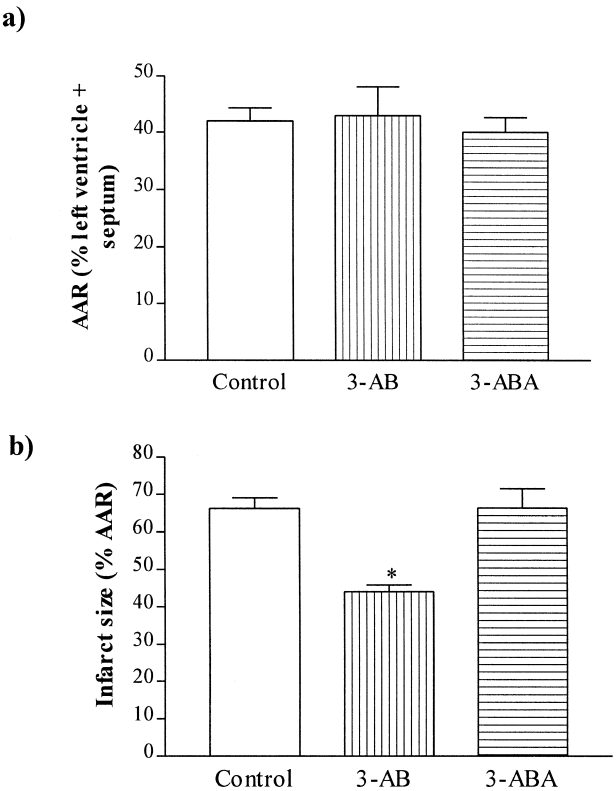

Fig. 1. (a) Area at risk (AAR) and (b) myocardial infarct size caused by occlusion of the coronary artery (1 h) followed by reperfusion (3 h) in the anaesthetised pig. Different groups of animals received (at 1 min prior to reperfusion) bolus injections of vehicle (saline-control, $n = 6$), the PARS inhibitor 3-aminobenzamide (3-AB, 10 mg/kg i.a., $n = 6$) or its inactive analogue 3-aminobenzoic acid (3-ABA, 10 mg/kg i.a., $n = 4$). Data are expressed as mean ± S.E.M. * $P < 0.05$ when compared to control.

3.2. Haemodynamic data

Mean baseline values (prior to starting the experiment, time 0) for mean arterial pressure and heart rate were similar in all groups investigated (Table 1). Coronary artery occlusion (for 60 min) resulted in a small fall in mean arterial pressure which was not greater than 10 mm Hg and was not different between groups (Table 1). During the subsequent reperfusion period (for 3 h), there was a further fall in mean arterial pressure in pigs which had been treated with the vehicle for 3-aminobenzamide (saline) (Table 1). When compared to the vehicle-treated control group, neither 3-aminobenzamide nor its inactive analogue 3-aminobenzoic acid had a significant effect on this fall in mean arterial pressure during reperfusion (Table 1). Myocardial ischaemia and reperfusion had no effect on heart rate and neither did any of the drugs investigated (Table 1).

Table 1
Mean arterial pressure and heart rate of pigs subjected to occlusion of the left anterior descending coronary artery (1 h) followed by reperfusion (3 h) in the anaesthetized pig

Group	Occlusion (min)					Reperfusion (min)						
	0	15	30	45	60	15	30	45	60	90	120	180
Control												
MAP (mmHg)	93 ± 7	84 ± 5	83 ± 5	84 ± 6	83 ± 7	78 ± 7	73 ± 8	71 ± 8	73 ± 8	72 ± 9	69 ± 8	60 ± 5
HR (beats/min)	98 ± 5	100 ± 6	103 ± 7	101 ± 7	104 ± 9	115 ± 9	127 ± 9	131 ± 11	131 ± 12	138 ± 15	137 ± 15	143 ± 4
3-aminobenzamide												
MAP (mmHg)	93 ± 4	86 ± 6	83 ± 7	86 ± 7	88 ± 7	80 ± 7	73 ± 8	72 ± 8	69 ± 8	72 ± 7	73 ± 6	74 ± 6
HR (beats/min)	93 ± 5	95 ± 7	98 ± 8	98 ± 7	99 ± 8	104 ± 7	116 ± 12	119 ± 13	121 ± 14	122 ± 15	128 ± 16	119 ± 14
3-aminobenzoic acid												
MAP (mmHg)	91 ± 6	84 ± 1	93 ± 1	91 ± 1	93 ± 3	83 ± 5	76 ± 5	66 ± 1	73 ± 5	63 ± 6	61 ± 4	55 ± 3
HR (beats/min)	103 ± 3	105 ± 2	106 ± 2	105 ± 2	105 ± 2	130 ± 11	148 ± 15	159 ± 17	163 ± 19	169 ± 19	171 ± 21	163 ± 41

Different groups of animals received (at 1 min prior to reperfusion) bolus injections of vehicle (control, $n = 6$), the PARS inhibitor 3-aminobenzamide (10 mg/kg i.a., $n = 6$) or its inactive analogue 3-aminobenzoic acid (10 mg/kg i.a., $n = 4$). Data are expressed as mean ± S.E.M. $* P < 0.05$ when compared to control.

Mean baseline data for myocardial contractility (dp/dt_{max}) (Control: 1750 + 136 mmHg/s, 3-aminobenzamide: 1383 + 164 mmHg/s, 3-aminobenzoic acid: 1688 + 171 mmHg/s) were not significantly different between groups. Coronary artery occlusion resulted in a 16 to 20% fall in dp/dt_{max}. There was no difference in dp/dt_{max} prior to the administration of vehicle or drugs between any of the groups studied ($P > 0.05$). In vehicle-treated, control animals, reperfusion was associated with a progressive decline in left ventricular contractility (Fig. 2). Administration of the PARS inhibitor 3-aminobenzamide attenuated this fall in dp/dt_{max} (Fig. 2). In contrast, the inactive analogue of this PARS inhibitor, 3-aminobenzoic acid, was without effect on the fall in dp/dt_{max} which occurred during the reperfusion period (Fig. 2).

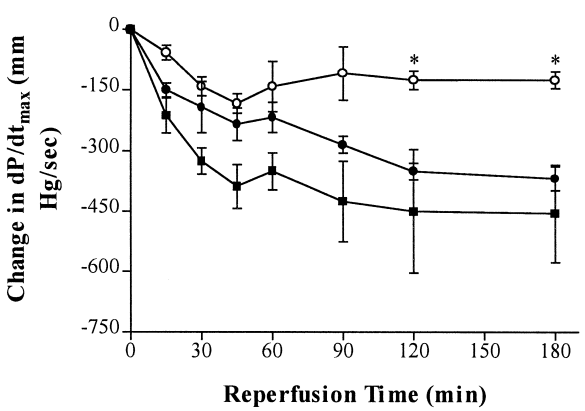

Fig. 2. Changes in myocardial contractility (measured as change in dp/dt_{max}) during the reperfusion period following occlusion of the coronary artery (1 h) in the anaesthetised pig. Different groups of animals received (at 1 min prior to reperfusion) bolus injections of vehicle (saline-control, closed circles, $n = 6$), the PARS inhibitor 3-aminobenzamide (3-AB, 10 mg/kg i.a., open circles, $n = 6$) or its inactive analogue 3-aminobenzoic acid (3-ABA, 10 mg/kg i.a., closed squares, $n = 4$). Data are expressed as mean ± S.E.M. $* P < 0.05$ when compared to control.

3.3. Cell injury and death caused by hydrogen peroxide in human cardiomyoblasts

Exposure of human cardiomyoblasts to hydrogen peroxide (4 h) caused a concentration-dependent reduction in mitochondrial respiration and increase in lactate dehydrogenase release into the cell supernatant (necrosis) (Fig. 3). Exposure of human cardiomyoblasts to hydrogen peroxide (3 mM for 4 h) caused a fall in mitochondrial respiration, and the release of LDH into the culture medium (Fig. 4). Pretreatment of these cells with 3-aminobenzamide (3 mM) significantly reduced both the impairment in mitochondrial respiration (Fig. 4a) as well as the cell death (release of LDH) caused by hydrogen peroxide (Fig. 4b). When compared to 3-aminobenzamide (3 mM), 3-aminobenzoic acid (3 mM) did neither attenuate the cell injury (impairment in mitochondrial respiration) nor the cell death

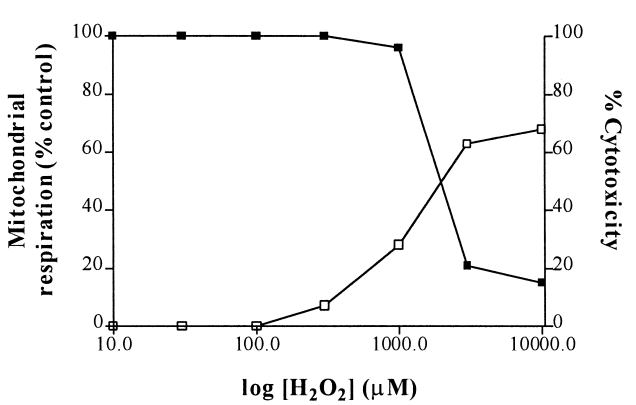

Fig. 3. Exposure of human cardiomyoblasts to hydrogen peroxide caused a concentration-dependent reduction in mitochondrial respiration (closed symbols, $n = 4$) and an increase in the release of lactate dehydrogenase into the supernatant (an indicator of cell necrosis, expressed as percent cytotoxicity) (open symbols, $n = 4$). Data are expressed as mean ± S.E.M. of n observations.

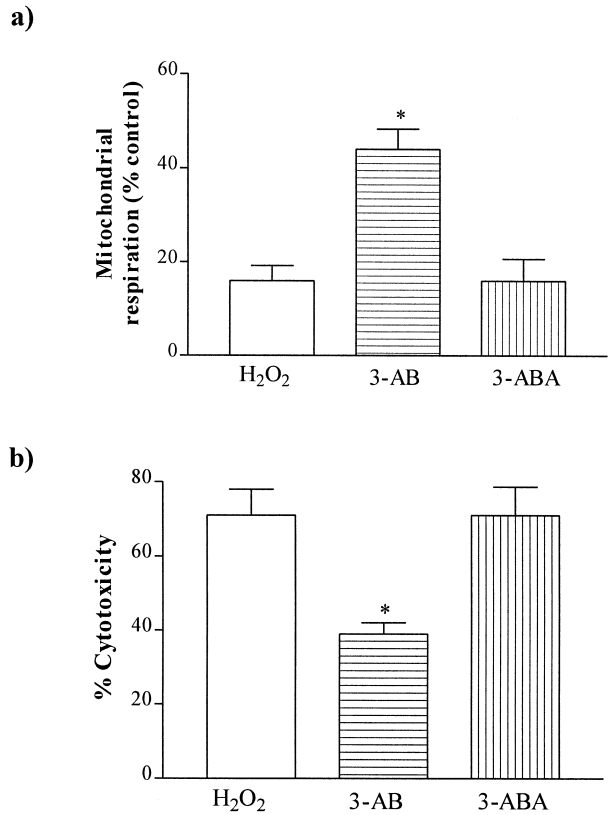

Fig. 4. The effect of the PARS inhibitor, 3-aminobenzamide (3-AB) on (a) the impairment in mitochondrial respiration and (b) the necrosis (measured as release of LDH into the cell supernatant) caused by hydrogen peroxide (H_2O_2, 3 mM, $n=6$) in human cardiomyoblasts. Preincubation of cells with 3-AB (3 mM, $n=6$), but not with its inactive analogue 3-aminobenzoic acid (3-ABA, 3 mM, $n=6$) reduces the impairment in mitochondrial respiration as well as the cell necrosis caused by hydrogen peroxide Data are expressed as mean ± S.E.M. $* P < 0.05$ when compared with H_2O_2-control (solid column).

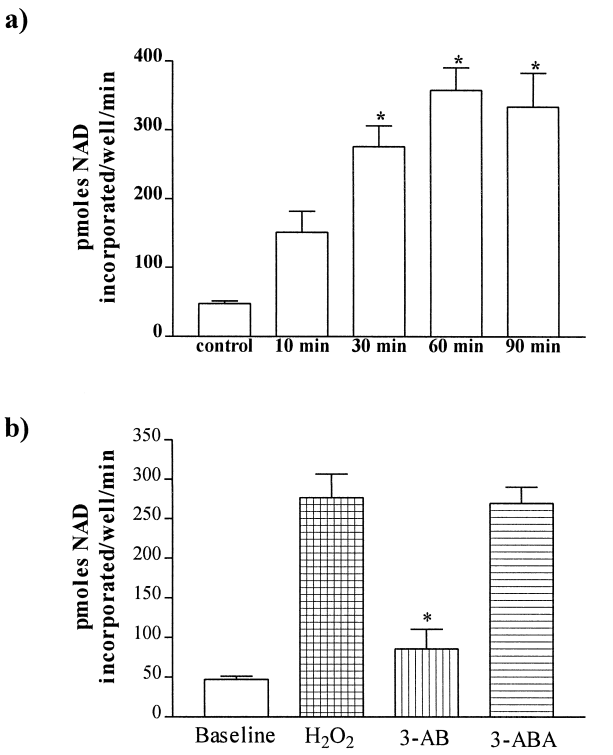

Fig. 5. The effect of preincubation of the PARS inhibitor, 3-aminobenzamide (3-AB) on the increase in PARS activity caused by hydrogen peroxide in human cardiomyoblasts. (a) Hydrogen peroxide (3 mM) caused a time-dependent increase in PARS activity ($n=6$). (b) The PARS inhibitor 3-AB (3 mM, $n=6$) attenuated the increase in PARS activity caused by H_2O_2 (30 min). 3-aminobenzoic acid (3-ABA, 3 mM, $n=6$) did not have any effect. Data are expressed as mean ± S.E.M. of n observations $* P < 0.05$ when compared with H_2O_2-control.

caused by hydrogen peroxide in human cardiomyoblasts (Fig. 4).

3.4. Effect of hydrogen peroxide on PARS activity in human cardiomyoblasts

Exposure of human cardiomyoblasts to hydrogen peroxide (3 mM) caused a time-dependent increase in PARS activity (Fig. 5a). This increase in PARS activity caused by hydrogen peroxide at 60 min was attenuated by pretreatment of these cells with 3-aminobenzamide (Fig. 5b). In contrast, 3-aminobenzoic acid did not affect the rise in PARS activity caused by hydrogen peroxide (Fig. 5b).

Exposure of human cardiomyoblasts to hydrogen peroxide (3 mM for 60 min) caused a maximal increase in PARS activity. Addition of 3-aminobenzamide to the permeabilised cells abolished the rise in PARS activity caused by hydrogen peroxide (Fig. 6). In contrast, 3-aminobenzoic acid did not affect PARS activity even when added to permeabilised cells (Fig. 6).

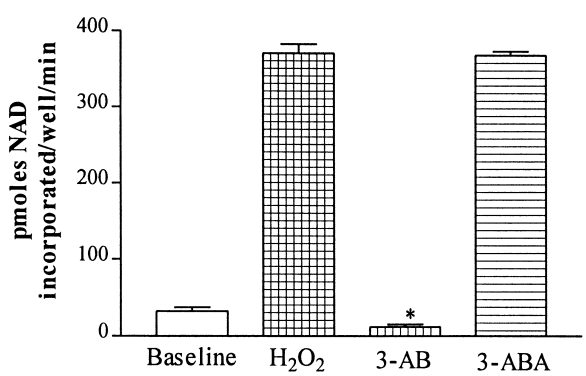

Fig. 6. The effect of the PARS inhibitor, 3-aminobenzamide when added to permeabilised human cardiomyoblasts on the increase in PARS activity caused by hydrogen peroxide. Hydrogen peroxide (3 mM) caused a significant increase in PARS activity ($n=6$). Addition of 3-aminobenzamide (3-AB, 3 mM, $n=4$) abolished the increase in PARS activity caused by hydrogen peroxide, but 3-aminobenzoic acid (3-ABA, 3 mM, $n=4$) did not have any effect. Data are expressed as mean ± S.E.M. of n observations $* P < 0.05$ when compared with H_2O_2-control.

4. Discussion

We have recently discovered that administration prior to reperfusion of various PARS inhibitors including 3-aminobenzamide reduce the degree of infarction caused by regional or global myocardial ischaemia in the rabbit, and the contractile dysfunction caused by global ischaemia in the isolated, buffer-perfused heart of the rabbit (Thiemermann et al., 1997). The degree of the reduction in infarct size in afforded by 3-aminobenzamide was similar when the PARS inhibitor was given either prior to ischaemia and prior to reperfusion, prior to ischaemia only or prior to reperfusion only. Thus, we have proposed (Thiemermann et al., 1997) that the observed reduction in infarct size is due to attenuation of reperfusion injury rather than ischaemic injury. Here we report that 3-aminobenzamide also causes a substantial reduction in infarct size when given prior to reperfusion following regional myocardial ischaemia in the anaesthetised pig. In addition, 3-aminobenzamide attenuated the contractile dysfunction (i.e., the decline in dp/dt_{max}) associated with regional myocardial ischaemia and reperfusion in this species. Clearly, this finding demonstrates that a substantial degree of the tissue injury associated with myocardial ischaemia-reperfusion occurs during the reperfusion period (i.e., reperfusion injury).

It could be argued that the cardioprotective effects of 3-aminobenzamide are due to nonspecific effects of this agent (rather than its ability to inhibit PARS activity). This is, however, not the case, as 3-aminobenzoic acid, the structure of which is very similar to the one of 3-aminobenzamide, but which does not inhibit PARS activity, did not reduce infarct size or affect the postischaemic contractile dysfunction. This study also demonstrates that exposure of human cardiomyoblasts to hydrogen peroxide for 4 h causes a concentration-dependent fall in mitochondrial respiration and the release of the cytosolic enzyme LDH into the supernatant (cell necrosis). Thus, hydrogen peroxide caused within 4 h an impairment in mitochondrial function and the death of human cardiomyoblasts. There is good evidence that (i) oxygen-derived free radicals cause strand breaks in DNA (Okamoto et al., 1996) and activation of PARS in many cell types including rat cardiomyoblasts (Gilad et al., 1997; Bowes et al., 1998), and (ii) strand breaks in DNA activate PARS (Schraufstatter et al., 1986b). We report here that pretreatment of human cardiomyoblasts with 3-aminobenzamide attenuates both the impairment in mitochondrial respiration as well as the cell necrosis caused by hydrogen peroxide. We, therefore propose that the activation of PARS contributes to the cell injury/necrosis caused by hydrogen peroxide in human cardiomyoblasts. This hypothesis is supported by the following key findings. (1) Exposure of human cardiomyoblasts to hydrogen peroxide caused within 60 min a maximal increase in PARS activity. (2) This increase in PARS activity was attenuated by 3-aminobenzamide. (3) At a concentration (3 mM) which attenuated the increase in PARS activity, 3-aminobenzamide caused a significant reduction in the impairment in mitochondrial function and cell necrosis caused by hydrogen peroxide (4) 3-aminobenzoic acid did neither inhibit the increase in PARS activity nor the cell injury/necrosis caused by hydrogen peroxide (negative control). Interestingly, pretreatment of human cardiomyoblasts (i.e., with intact cell membranes) with 3 mM of 3-aminobenzamide resulted in a 70% reduction in PARS activity. Thus, one could argue that 3-aminobenzamide is a relatively weak inhibitor of PARS activity. This is, however, not the case, as the addition of 3-aminobenzamide (3 mM) to permeabilised cells abolished PARS activity (i.e., reduction in PARS activity below baseline). Thus, we propose that the inhibition of PARS activity afforded by 3-aminobenzamide in whole cells and animals is limited by the ability of this compound to cross cell membranes.

The mechanism by which inhibitors of PARS activity reduce infarct size in the heart and other organs is largely unclear. This study demonstrates that inhibition of PARS activity with 3-aminobenzamide protects human cardiomyoblasts against hydrogen peroxide suggesting that PARS inhibitors can directly protect cardiac myocytes. This is also supported by our previous finding that 3-aminobenzamide reduces the infarct size caused by ischaemia-reperfusion of the isolated, buffer-perfused heart of the rabbit (Thiemermann et al., 1997). In addition to the generation of oxygen-derived free radicals, the infiltration and activation of neutrophils contributes to reperfusion injury of the heart (Lucchesi et al., 1989). There is evidence that inhibitors of PARS activity modulate neutrophil extravasation. For instance, in the perfused mesentery of the mouse, 3-aminobenzamide significantly reduces the number of cells that emigrated outside the postcapillary venules without modifying the extent of neutrophil adhesion (Szabo et al., 1997). In addition, the reduction in myocardial infarct size afforded by 3-aminobenzamide in the rat is associated with a reduced accumulation of neutrophils in the ischaemic-reperfused myocardium (measured as myeloperoxidase activity) (Zingarelli et al., 1997). Thus, it is possible that prevention of the infiltration of neutrophils may have contributed to the cardioprotective effects of 3-aminobenzamide. However, the reduction of infarct size afforded by 3-aminobenzamide is very similar in (i) rabbit (Thiemermann et al., 1997) and pig hearts (this study) subjected to regional myocardial ischaemia, (ii) the isolated, buffer-perfused heart of the rabbit subjected to global ischaemia (Thiemermann et al., 1997), and (iii) the isolated, buffer-perfused heart of the rat subjected to regional myocardial ischaemia (Bowes et al., 1998). Thus, it appears that a substantial part of the cardioprotective effects of PARS inhibitors is independent of any effects of these agents on neutrophils. It should be noted that the cardioprotective effects of 3-aminobenzamide is also unlikely to be due to the ability of this agent to scavenge

oxygen-derived free radicals, as 3-aminobenzamide (at concentrations which inhibited PARS activity and prevented cell injury/death in human cardiomyoblasts) did not scavenge superoxide anions (Bowes et al., 1998).

In conclusion, this study demonstrates that the PARS inhibitor 3-aminobenzamide (but not its inactive analogue 3-aminobenzoic acid) caused a substantial reduction in reperfusion injury caused by regional myocardial ischaemia-reperfusion in the anaesthetised pig. Similarly, 3-aminobenzamide (but not its inactive analogue) attenuated the cell injury/necrosis as well as the increase in PARS activity caused by hydrogen peroxide in human cardiomyoblasts. These 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 reactive oxygen species. We propose that 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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