Evidence that cyclophilin-A protects cells against oxidative stress

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Cyclophilin-A is the cytosolic isoform of a family of peptidyl-proline *cis–trans*-isomerases that bind cyclosporin A. This study investigates the role of cyclophilin-A in necrotic cell death, induced by 'chemical ischaemia' and by t-butylhydroperoxide. An 18-mer antisense phosphorothioate oligodeoxynucleotide was used to target a translated region of cyclophilin-A mRNA in rat neonatal cardiomyocytes. After a 24 h exposure to the oligonucleotide, the amount of cyclophilin-A in the cells was decreased by at least 93% as judged by immunological and enzymic criteria. For the enzyme assays, peptidyl proline *cis–trans*-isomerase activity was measured fluorimetrically in small (10 μl) volumes of cell extract. Immunoblots were developed with a polyclonal anti-cyclophilin-A antibody after sample isoelectric focusing and SDS/PAGE. Cyclophilin-A suppression had no effect on cyanide-plus-2-deoxyglucose-induced cell death. However, cyclophilin-A-suppressed cells were markedly more sensitive to t-butylhydroperoxide. Cyclosporin A conferred some resistance to the peroxide in both types of cell, but protection was greater in cyclophilin-A-suppressed cells, where cyclosporin A increased the survival time 2-fold. It is concluded that two cyclophilin isoforms are involved, in quite different ways, in peroxide-induced cell death. Cyclophilin-A has a protective role. Another isoform, possibly mitochondrial cyclophilin-D, has a deleterious role, such that blockade by cyclosporin A leads to protection.

Key words: antisense oligodeoxynucleotides, cardiomyocytes, cyclosporin A.

INTRODUCTION

The cyclophilins (CyPs) form a family of cyclosporin A (CSA)-binding proteins that catalyse rotation of Xaa–Pro peptide bonds. CSA binds to the active site and blocks peptidyl proline *cis–trans*-isomerase (PPIase) activity. CyPs have been isolated from a wide variety of organisms. In mammalian tissues distinct isoforms are found in different intracellular compartments e.g. CyP-A (cytosol), CyP-B and CyP-C (endoplasmic reticulum) and CyP-D (mitochondria) [1–5]. The physiological functions of CyPs are not well understood. Their isomerase activity suggests a role in catalysing protein folding and/or conformational changes. *In vitro*, CyPs catalyse the refolding of some denatured proteins [6,7] and they appear to catalyse *de novo* folding of some proteins, at least, *in vivo*, e.g. transferrin [8], rhodopsin in *Drosophila* [9] and mitochondrial imported proteins in yeast [10]. CyPs also participate in larger protein complexes, although the functional significance has yet to be resolved. Complexes include the oestrogen receptor (CyP-40, a CyP-domain protein) [11], the nuclear pore complex (Nup358, a CyP-domain protein) [12], the calcium-modulating cyclophilin ligand ('CAML')–Ca^{2+}-ATPase–calreticulin complex of sarcoplasmic reticulum (CyP-B) [13] and the mitochondrial voltage-dependent anion channel (VDAC)–adenine nucleotide translocase (ANT) complex (CyP-D) [14].

The subject of the present study, CyP-A, binds to the heat shock protein hsp90 [15] and to the antioxidant protein Aop1 [16]. In addition the CyP-A–CSA complex binds strongly to calcineurin and inhibits its Ca^{2+}-dependent protein phosphatase activity. Inhibition of calcineurin in T-lymphocytes leads to decreased nuclear levels of the transcription factor NF-AT, and this prevents T-cell activation; these events are believed to be responsible for the immunosuppressive action of CSA [17].

There are now numerous reports that CSA retards the onset of cell death in cardiomyocytes, hepatocytes and neurons brought on by ischaemia (or anoxia), reperfusion (reoxygenation) and pro-oxidants (see the Results and discussion section). However, in view of the diverse CyPs and CyP-domain proteins in cells, and the fact that their functions are poorly defined, precise interpretations often cannot be made. There are indications that, in hepatocytes and cardiomyocytes, CSA protection may reflect inhibition of the permeability transition (PT) pore [18–22]. The PT pore forms in the mitochondrial inner membrane from the VDAC–ANT–CyP-D complex ([14] and references cited therein) and is blocked by CSA [23]. However, in neurons, it is clear that calcineurin inhibition is the key aspect of CSA protection, which implicates CSA binding to CyP-A [24]. In the present study, we have used antisense-oligodeoxynucleotides (ODNs) against CyP-A to attenuate expression of this isoform in neonatal cardiomyocytes and analysed CSA protection in these cells. The data are consistent with a non-CyP-A target of CSA, possibly CyP-D. At the same time, we report a novel capacity of CyP-A to protect cardiomyocytes against oxidative stress as induced by t-butylhydroperoxide.

MATERIALS AND METHODS

Cell culture and assay

Primary cultures of cardiomyocytes were prepared as described by Fluri et al. [25] with modifications. Routinely, four 2-week-old Sprague–Dawley rats were anaesthetized with diethyl ether, anti-coagulated with heparin (250 units) and killed after 10 min. The hearts were chopped and incubated in Hanks balanced salt solution (Gibco-BRL), containing: 0.8 mg/ml collagenase type II, 20 μg/ml DNase II, 20 units/ml penicillin, 20 μg/ml streptomycin, 1 mM taurine and 40 μM $CaCl_2$ and incubated for

Abbreviations used: CyP, cyclophilin; CSA, cyclosporin A; PPIase, peptidyl proline *cis–trans*-isomerase; Aop, antioxidant protein; ODN, oligodeoxynucleotide; VDAC, voltage-dependent anion channel; ANT, adenine nucleotide translocase; PT, permeability transition; IEF, isoelectric focusing.

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15 min at 35 °C. This procedure was repeated three times, each time with fresh medium. After gentle centrifugation, cells (approx. 10^7) were resuspended in M199 culture medium (Sigma) containing: 20 units/ml penicillin, 20 μg/ml streptomycin, 2 μg/ml vitamin B_{12} and 10 % (v/v) foetal-calf serum. The cells were seeded on laminin-treated glass coverslips at a density of 5×10^5 cells/0.5 ml and incubated in an atmosphere of CO_2/air (1:19) at 37°C. After 3 h, 1 μM cytosine arabinoside was added to limit the growth of fibroblasts [26]. The following day cells were washed with saline, and fresh culture medium was added with or without 3 μM phosphorothioate antisense ODN. Cultures generally contained greater than 90 % cardiomyocytes. Cell viability was assayed in a solution containing: 100 mM NaCl, 4 mM KCl, 24 mM Hepes, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM KH_2PO_4 and 11 mM glucose, pH 7.4. The fluorescent, membrane-impermeant ethidium homodimer (2 μg/ml) was added to stain the nuclei of dead cells, and cells were viewed under a fluorescence microscope. Further additions are given in the Figure legends. Cells for immunodetection and enzyme assay (below) were washed in 150 mM NaCl/10 mM Hepes, pH 7.4, and then scraped off in the same medium. After centrifugation, cell pellets were stored at −70°C.

Immunodetection of CyP-A

Cell extracts were fractionated by isoelectric focusing (IEF) [27] and SDS/PAGE before blotting and immunodetection. IEF gels contained 5 % (w/v) acrylamide, 0.16 % (w/v) bisacrylamide, 4 M urea and 2.4 % (v/v) ampholytes (pH 7–9, 1 %; pH 9–11, 1 %; pH 3–10, 0.4 %). Cell pellets were extracted with 4 M urea/10 mM Bis-Tris propane, pH 6.5. Extracts were freed of DNA on DEAE-Sepharose (pH 6.5) and mixed with an equal volume of sample loading buffer [4 M urea/2.4 % (v/v) ampholytes/2 % (v/v) Triton X-100/1 % (v/v) mercaptoethanol/0.4 % Bromophenol Blue]. Gels were focused for 30 min at 150 V and a further 90 min at 200 V. The pH gradients were determined by soaking 1 cm gel slices in 1 ml of 10 mM KCl for 30 min. The positions of the CyP-A and CyP-D markers were determined by fixing the gels in 10 % (w/v) trichloroacetic acid (10 min) followed by 1 % (w/v) trichloroacetic acid (2 h) to remove ampholytes, and staining with Coomassie Brilliant Blue. CyP-A and CyP-D (for use as markers) were purified from rat liver to single bands on SDS/PAGE as described [5]. Horizontal IEF gel strips were subjected to SDS/PAGE in the second dimension. The strips were equilibrated (1 h) in 62 mM Tris/HCl (pH 6.8)/5 % (v/v) 2-mercaptoethanol/ 2 % (w/v) SDS/10 % (v/v) glycerol, overlaid on the stacking gel and electrophoresed as described previously [28]. Proteins separated by SDS/PAGE were electroblotted on to 0.45-μm pore-size nitrocellulose membranes in 20 % (v/v) methanol/25 mM Tris/HCl/192 mM glycine, pH 8.3 (1 h, 50 V). Membranes were blocked with 150 mM NaCl/ 10 mM sodium phosphate (pH 7.4)/0.1 % (v/v) Tween 20 ($NaCl/P_i$/Tween) containing 5 % (w/v) milk powder, then washed three times in $NaCl/P_i$/Tween and incubated with rabbit anti-CyP-A antibody (1:1000) (Calbiochem) for 1 h. The membranes were washed again three times in $NaCl/P_i$/Tween and incubated in peroxidase-labelled goat anti-rabbit IgG antibodies for 1 h. After five further washes, bands were developed with the Enhanced Chemiluminescence kit from Amersham.

Enzymic detection of CyP-A

Cell pellets were homogenized on ice in 210 mM mannitol/ 70 mM sucrose/10 mM Tris/HCl (pH 7.4)/50 mM KCl containing the protease inhibitors antipain, chymostatin, pepstatin, leupeptin and aprotinin (all at 1 μg/ml). Extracts were centrifuged in a Beckman Airfuge (130000 ***g***, 10 min). Supernatants (cytosolic fraction) were assayed for PPIase (CyP-A) using the fluorescent substrate *N*-succinyl-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin, by the method of Kofron et al. [29] and as described previously [30]. Fluorescence was measured with an excitation wavelength of 480 nm and emission at > 510 nm in a 10 μl reaction chamber on the stage of a fluorescence microscope. The microscope was equipped with a side-mounted photomultiplier tube enabling the relatively small amounts of cytosolic fraction to be assayed [30].

RESULTS AND DISCUSSION

Suppression of CyP-A expression

CyP-A (18 kDa) is resident in the cytosol. Mammalian tissues contain several other CyPs with distinct intracellular locations, i.e. CyP-B (20–22 kDa, endoplasmic reticulum [1,2]), CyP-C (23 kDa, endoplasmic reticulum [3]), CyP-D (19 kDa, mitochondria [4,5]) as well as the CyP-domain proteins, CyP-40 (40 kDa [31]) and Nup358 [12]. To investigate the function of CyP-A, the principal requirement was an antisense ODN for the selective suppression of CyP-A translation, only. There are no general rules for which part of the mRNA offers the most effective target for inhibition of protein expression by antisense ODNs; effective antisense ODNs have been targeted against both the translated and untranslated regions of the mRNA ([32] and references cited therein). We opted for an 18-base region towards the 3′ end of the translated region of CyP-A cDNA (492 bp) which was absent from CyPs B, C and D (Figure 1) and from the CyP-domain proteins CyP-40 [31] and Nup358 [12].

For immunological evaluation of CyP-A expression, we used an anti-CyP-A polyclonal antibody which had slight reactivities towards CyPs B and D. In practice, the cross-reactivity of the antibody was negligible, in particular, since CyP-A seems to be a major isoform in heart cells. The relative amount of CyP-A was estimated from the CSA-sensitive PPIase activity (see below) in the cytosol (CyP-A) and particulate fractions (CyPs B, C and D together) of cultured neonatal myocytes. The cytosol/particulate PPIase activity ratio was about 3:1. In addition, Western blots (after SDS/PAGE) of the particulate fraction contained no visible antigenic material around 20 kDa under standard conditions. Nevertheless, to ensure that CyP-A was measured specifically, cell extracts were routinely subjected to IEF before SDS/PAGE (and blotting). CyP-A is the least basic of the 18–23 kDa CyPs. It can, therefore, be separated from CyP-D by

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CyP-A                AGCATTGTGGAAGCCATG

Antisense ODN  3'- TCGTAACACCTTCGGTAC - 5'
Nonsense  ODN  5'- TCGTAACACCTTCGGTAC - 3'

CyP-B                GATGTGGTACGGAAGGTG
CyP-C                ACTGTGGTACATTCCATT
CyP-D                GATGTTGTGAAGAAAATA
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Figure 1 Antisense ODN used in this study

The relevant sequence of the translated region of rat CyP-A cDNA [52] is shown along with the corresponding phosphorothioate ODNs. For comparison, the equivalent regions of cDNA for rat CyP-B [53] and CyP-D [54], and mouse CyP-C [3], are also given.

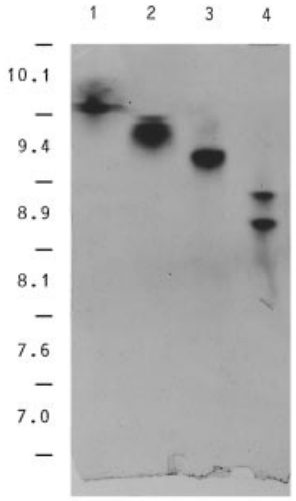

Figure 2 Isoelectric focusing of CyP-A

Purified rat CyP-A (lane 3) was run alongside purified rat CyP-D (lane 2) and the markers cytochrome *c* (lane 1) and lentil lectin (lane 4), as described in the Materials and methods section. The pH profile (7.0–10.1) is also given on the left-hand side.

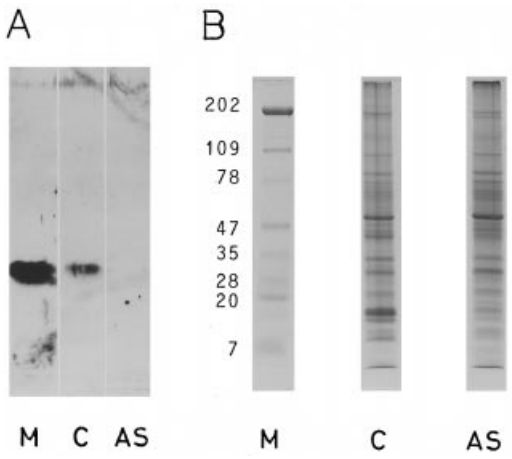

Figure 3 Antisense ODN suppression of CyP-A as detected immunologically

(**A**) Extracts of myocytes (10^5 cells) were fractionated by IEF and the band corresponding to CyP-A subjected to SDS/PAGE. Western blots were then developed with anti-CyP-A antibodies. Lanes: C, control myocytes; AS, antisense-ODN-treated (24 h) myocytes; M, purified CyP-A marker (1 μg). (**B**) Myocyte extracts were analysed by SDS/PAGE (Coomassie Blue-stained). Lanes: C, control myocytes; AS, antisense-ODN-treated (24 h) myocytes; M, molecular-mass markers (kDa).

IEF as shown in Figure 2. CyP-A is similarly separated by IEF from CyP-B ([2,31] and confirmed in this laboratory). In practice cell extracts were freed of DNA and then fractionated by IEF alongside CyP-A and CyP-D markers. After IEF, horizontal strips containing CyP-A, but not CyP-D, were cut out and subjected to SDS/PAGE, before blotting and analysis.

Figure 3(A) shows immunoblots of cell extracts from myocytes treated with the antisense ODN against CyP-A. Whereas control extracts yielded a single immunogenic band corresponding to CyP-A, this band was absent after 1 day of exposure to the antisense ODN. Within the limits of detection, therefore, ex-

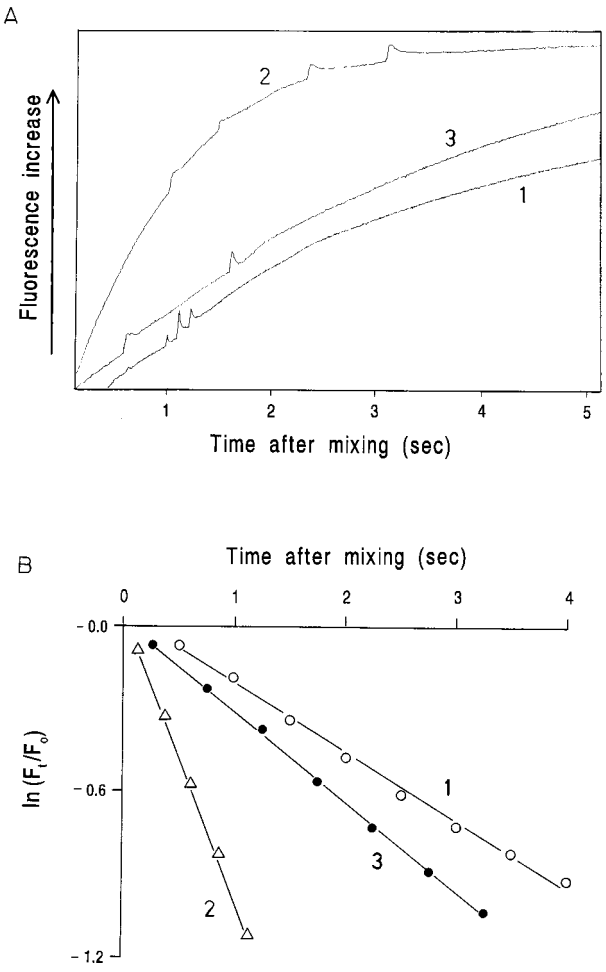

Figure 4 Antisense ODN suppression of CyP-A as detected enzymically

(**A**) Cytosolic extracts of myocytes (10^5 cells) were assayed for PPIase activity: trace 1, no extract; trace 2, control myocytes; trace 3, antisense-ODN-treated (24 h) myocytes. (**B**) Data are replotted according to the relationship: $\ln(F_t/F_0) = -kt$ where F_t is the fluorescence change that occurred subsequent to any time t and F_0 is the maximal fluorescence change recorded.

pression of CyP-A was effectively suppressed. The nonsense ODN (Figure 1) did not visibly affect CyP-A expression (results not shown). Antisense ODNs are statistically unlikely to select one target mRNA species. To gain some idea of the extent of protein suppression, Coomassie Blue-stained gels of the cytosol of control and antisense-ODN-treated cells were compared (Figure 3B). The general pattern of protein expression was the same with and without antisense ODN. There was a major depleted band at about 18 kDa, corresponding to CyP-A, but whether this is indeed CyP-A is not known. Some other bands were also absent after antisense ODN treatment (e.g. 12 kDa). Thus the antisense ODN was largely selective, but not completely so. Experiments (not shown) in which lower concentrations of the antisense ODN (1 μM) were used did not visibly yield better selective suppression of CyP-A and did not always produce as efficient CyP-D suppression. Under standard conditions of culture, the antisense ODN had no effect on the morphology and viability of the cardiomyocytes.

In order to quantify the losses of CyP-A brought about by antisense ODN treatment, PPIase assays were performed on

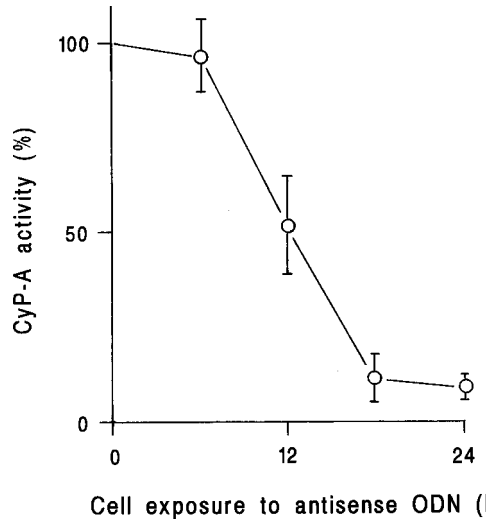

Figure 5 Time-dependence of antisense ODN suppression of CyP-A

Cells were exposed to antisense ODN for the time indicated before extraction; control cells (minus antisense ODN) were cultured and extracted in parallel. Cytosolic extracts were assayed for PPIase activity as described in the legend to Figure 4(B). Values are means ± S.E.M. ($n = 3$).

cytosolic fractions of cell extracts. The assay employed a test peptide, which is hydrolysed by chymotrypsin strictly when the Ala–Pro bond is in the *trans* configuration, resulting in the release of fluorogen. At the high concentrations of chymotrypsin used, pre-existing *trans* peptide is hydrolysed within the mixing time and is not recorded, but further hydrolysis requires *cis–trans* isomerization. Uncatalysed *cis–trans* isomerization occurs, and this limits the sensitivity of the technique (Figure 4A, trace 1). Nevertheless, the rate of isomerization was greatly increased by the cytosol fraction of control cells (Figure 4A, trace 2). CSA inhibited $> 90\,\%$ of the increment in the rate of isomerization due to the cytosol (results not shown), confirming that it reflected CyP-A activity. In contrast, an equivalent amount of cytosol from antisense-ODN-treated cells yielded little increase in rate of isomerization (Figure 4A, trace 3). This is consistent with near complete suppression of CyP-A in antisense-ODN-transfected cells.

The PPIase data are best analysed from semi-logarithmic plots (Figure 4B), allowing the pseudo-first-order rate constants (negative slopes) to be determined. After correction for the basal, uncatalysed rate ($0.26\ s^{-1}$), the cytosolic PPIase activity of antisense-ODN-treated cells ($0.07\ s^{-1}$ per 10^5 cells) amounted to $9\,\%$ of that of the control cells ($0.76\ s^{-1}$ per 10^5 cells) in this particular pair of test and control. In three separate experiments CyP-A activity, after antisense ODN treatment, amounted to $8 \pm 3\,\%$ (mean ± S.E.M.) of that in the control cells. In Figure 5, the antisense ODN was applied for various times before extraction and assay of cytosolic PPIase activity. About 12 h was required for $50\,\%$ decrease in CyP-A. Thus after 24 h treatment with antisense ODN, CyP-A was substantially decreased as judged by both immunological and enzymic criteria.

Effect of CyP-A suppression on cell death

The antisense ODN described (Figure 1) provides a tool for investigating CyP-A functions in cells which, at present, are poorly understood. Here we have used it to probe the role of CyP-A in the pathogenesis of necrotic cell death. This follows from the documented ability of CSA to retard the onset of necrotic cell death induced by anoxia/reoxygenation [18–20,33,34] and added peroxides [35–37] in heart cells and hepatocytes. These observations of CSA protection point to an involvement of a CyP in ischaemia-and-reperfusion-induced cell death, but provide no indication of which particular CyP isoform is involved. We have used two protocols to investigate this problem. First, myocyte cell death was induced by cyanide and deoxyglucose, which blocks both aerobic and anaerobic catabolism, and mimics ischaemia. In the second protocol, cell death was brought about by oxidative stress induced by t-butylhydroperoxide. This treatment mimics the oxidative stress associated with reperfusion-induced cell death, when superoxide radical ($O_2^{\bullet -}$) and H_2O_2 are believed to arise at sites predominantly extracellular to the cardiomyocytes, i.e. endothelial cells (xanthine oxidase [38]), neutrophils and platelets [39,40]. In both protocols, a group of 6–8 well-separated myocytes were observed under the fluorescence microscope at 2 min intervals until nuclear staining by ethidium homodimer was just apparent. This was taken as the time of cell death. Tests (antisense ODN treatment) and controls were done on cells from the same culture. This procedure was repeated with five coverslips, giving a total of 37 cells for both tests and controls. Myocytes incubated without cyanide and deoxyglucose or peroxide were stable for at least 140 min as judged by the lack of nuclear staining by ethidium homodimer.

Figure 6(A) shows that cells died 50–130 min after exposure to cyanide and deoxyglucose. CyP-A suppression had no effect on the survival time under these conditions. On the other hand, cells lacking CyP-A were markedly more susceptible to t-butylhydroperoxide (Figure 6B); in this case, the survival time was approximately halved by CyP-A suppression (survival times, mean ± S.E.M.: Control, 94 ± 4 min; Antisense-ODN-treated, 56 ± 3 min). The pathogenesis of both forms of cell death have features in common, namely the loss of ATP and adenine nucleotides, as they are degraded to nucleosides and bases, and the rise in cell Ca^{2+} ([41–44] and references cited therein). These events, in turn, produce a spectrum of dysfunctions, culminating in cell death. However, the selective increase in sensitivity to peroxides suggests that CyP-A suppression had no primary effect on Ca^{2+} or energy metabolism. Instead, a specific correlation of CyP-A with peroxide or antioxidant metabolism is indicated.

A possible link between CyP-A and peroxide metabolism has recently emerged: organic hydroperoxides are reduced by GSH in a reaction catalysed by glutathione peroxidase. They also lead to increased O_2-derived free radicals, which generate free-radical sites in biological molecules. GSH can repair these sites with the generation of thiyl radicals ($RS^{\bullet}$), which may also be deleterious ([45] and references cited therein). A thiol-specific antioxidant protein has been identified in yeast that protects against t-butylhydroperoxide and H_2O_2 [46], possibly by catalysing the removal of thiyl radicals [45]. Related proteins with considerable sequence similarities are found in *Salmonella typhimurium* (C22 subunit of alkylhydroperoxide reductase [47]) and humans (Aop1 [48]). Expression of Aop1 in bacterial cells lacking the C22 subunit conferred resistance to t-butylhydroperoxide [48]. Aop1 is believed to perform a similar defensive role against peroxides in human cells. More recently it has been shown that human CyP-A binds strongly to human Aop1. This binding has been shown by employing the yeast two-hybrid system and by using a recombinant Cyp-A–(glutathione S-transferase) fusion protein as an affinity matrix [16]. In an *in vitro* system, CyP-A markedly increased the capacity of Aop1 to protect a test protein (glutathione synthetase) against thiol-specific oxidative inactivation [16]. The capacity of CyP-A to activate Aop1 was insensitive to CSA, indicating that the region involved in binding to Aop1 is

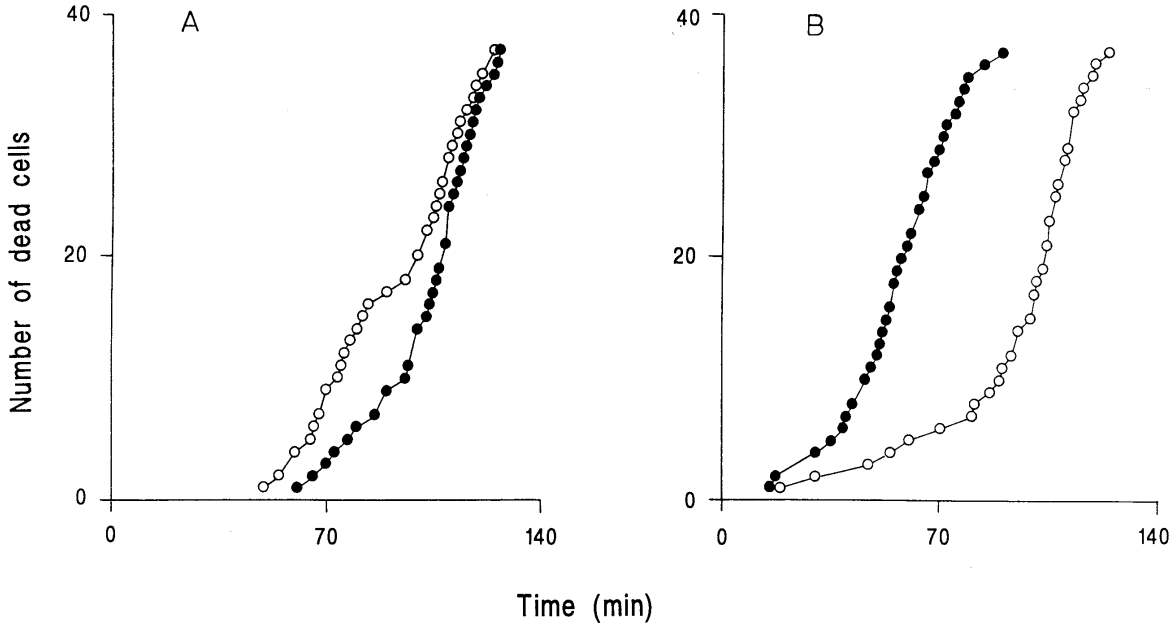

Figure 6 Effect of CyP-A suppression on the tolerance of myocytes to chemical ischaemia (A) and oxidative stress (B)

Control (○) and 24 h antisense-ODN-treated (●) myocytes were subjected to either 1 mM cyanide plus 20 mM 2-deoxyglucose (**A**) or to 500 μM t-butylhydroperoxide (**B**). The appearance of dead cells was quantified with time (see the Materials and methods section).

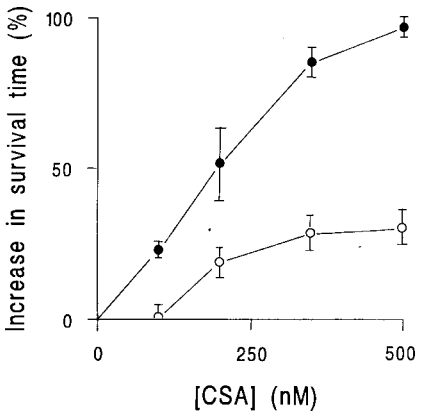

Figure 7 Effect of CSA on the tolerance of myocytes to oxidative stress

Control (○) and 24 h antisense-ODN-treated (●) myocytes were treated with 500 μM t-butylhydroperoxide in the presence of the CSA concentration indicated. Cells were preincubated with CSA for 10 min prior to peroxide addition. Values are means ± S.E.M. (four coverslips).

distinct from the active (CSA-binding) site [16]. It is possible, therefore, that the ability of CyP-A to protect against oxidant-mediated injury is brought about via interaction with Aop1.

There are numerous reports that CSA protects against injury involving oxidant stress (see above). Binding of CSA to CyP-A produces a complex that inhibits the Ca^{2+}/calmodulin-activated protein phosphatase, calcineurin, and CSA protection, in some models, is highly consistent with calcineurin inhibition (e.g. glutamate toxicity in neurons [24]). Other experimental models have implicated the mitochondrial isoform, CyP-D, as the primary target for protection. CyP-D controls the mitochondrial PT pore ([14] and references cited therein) which is blocked by CSA [23]. It was postulated that PT pore opening induced by Ca^{2+} and oxidative stress might provoke necrotic cell death during myocardial ischaemia/reperfusion [23,49–51], and various lines of evidence support this hypothesis [18–22]. In the present study we have used CyP-A-suppressed myocytes to pursue this question further.

Figure 7 shows the effect of CSA on the susceptibility of normal and CyP-A-suppressed myocytes to t-butylhydroperoxide. CSA prolonged the viability of normal, neonatal myocytes to a small extent. This degree of protection is much less than that observed previously using cardiomyocytes from adult rats [20]. Also in adult cells [20], as in perfused adult rat hearts [19], CSA yields a bell shaped protection curve with a maximum at 200 nM CSA, which was not observed in this case. However, the salient feature (Figure 7) is that CSA protection was actually greater in CyP-A-suppressed myocytes. This provides clear evidence that the protection by CSA in this particular model is not caused by binding to CyP-A. The data are consistent, at least, with CyP-D being the CSA target for protection.

In summary the present study provides evidence that two CyP isoforms are involved in peroxide-induced cell death, although in quite different ways. CyP-A in the cytosol has a protective function, possibly in association with Aop1. Conversely, another CyP has a deleterious role evident from the protection afforded by CSA in CyP-A-depleted cells. The identity of this isoform is not yet known, but it may be mitochondrial CyP-D, which activates the PT pore in a CSA-sensitive mannner. We are currently trying to suppress CyP-D to resolve this question.

This work was supported by the British Heart Foundation (PG/96118) and the Wellcome Trust.

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Received 29 January 1999/8 April 1999; accepted 30 April 1999