Protection from inactivation of the adenine nucleotide translocator during hypoglycaemia-induced apoptosis by mitochondrial phospholipid hydroperoxide glutathione peroxidase

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We demonstrated that mitochondrial phospholipid hydroperoxide glutathione peroxidase (PHGPx) first suppressed the dissociation of cytochrome *c* (cyt *c*) from cardiolipin (CL) in mitochondrial inner membranes and then apoptosis caused by the hypoglycaemia by the prevention of peroxidation of CL [Nomura, Imai, Koumura, Arai and Nakagawa (1999) *J. Biol. Chem.* **274**, 29294–29302; Nomura, Imai, Koumura, Kobayashi and Nakagawa (2000) *Biochem. J.* **351**, 183–193]. The present study shows the involvement of peroxidation of CL in the inactivation of adenine nucleotide translocator (ANT) and the opening of permeability transition pores by using the system of ANT-reconstituted liposome and isolated mitochondria. ANT activity appeared in dioleoyl phosphatidylcholine proteoliposome containing 10 % (mol/mol) CL or phosphatidylglycerol (PG), but not other classes of phospholipids. ANT activity was competitively inhibited by the addition of cardiolipin hydroperoxide (CLOOH) in reconstituted liposomes containing CL. However, phosphatidylcholine hydroperoxide failed to inactivate the activity of ANT. The activity of ANT in reconstituted liposomes, including CLOOH, recovered

when CLOOH in reconstituted liposome was reduced to hydroxycardiolipin by incubation with PHGPx. The activity of ANT was determined in rat basophil leukaemia RBL2H3 cells after their exposure to 2-deoxyglucose. ANT activity decreased to 50 % of the control level by 4 h in response to apoptosis. In parallel, cyt *c* and apoptosis-inducing factor (AIF) were released from mitochondria. Suppression of the accumulation of CLOOH by overexpression of PHGPx in mitochondria effectively prevented the inactivation of ANT, the opening of permeability transition pores and the release of cyt *c* and AIF from mitochondria in hypoglycaemia-induced apoptotic cells. These findings suggest that mitochondrial PHGPx might be involved in the modulation of the activity of ANT and the opening of pores for the release of cyt *c* via the modulation of levels of CLOOH in the mitochondria.

Key words: adenine nucleotide translocator, cardiolipin hydroperoxide, cytochrome *c* release, mitochondria, permeability transition pore complex.

INTRODUCTION

Mitochondria play a crucial role in apoptosis via the release of apoptotic factors such as cytochrome *c* (cyt *c*), apoptosis-inducing factor (AIF) and Smac/DIALBO from the intermembrane space into the cytosol [1]. The release of cyt *c* and AIF from mitochondria through permeability transition (PT) pores has been demonstrated in studies with inhibitors of the opening of such PT pores, for example, cyclosporin A (CsA) and certain apoptotic viral proteins [2]. The PT pores consist of proteins from both the inner mitochondrial membrane, including adenine nucleotide translocator (ANT), and the outer membrane, including the voltage-dependent anion channels (VDAC) and Bax/Bcl-2 family proteins [3]. A central role for ANT in the regulation of the opening of PT pores was demonstrated in experiments with two pharmacological inhibitors of ANT, bongkreikic acid (BA) and atractyloside (Atr), which interact with distinct binding sites on ANT and regulate, in different ways, the opening of pores that results in the release of cyt *c* [4,5]. BA suppresses the opening of PT pores and the release of cyt *c* from mitochondria. On the

other hand, Atr can induce the release of cyt *c* via the opening of PT pores. However, details of the mechanism of release of mitochondrial apoptotic factors, such as cyt *c*, from mitochondria and details of the regulation of ANT activity remain to be resolved.

Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is a unique intracellular antioxidant enzyme that directly reduces peroxidized phospholipids that have been produced in cell membranes [6,7]. PHGPx exists in both mitochondrial and non-mitochondrial forms [8,9]. The two forms of PHGPx play several independent and important roles in the modulation of inflammation, spermatogenesis and cell death [7,10–16]. We have shown previously that overexpression of mitochondrial PHGPx in rat basophil leukaemia cells (RBL2H3 cells) suppressed the release of cyt *c* from mitochondria, as well as apoptosis induced by 2-deoxyglucose (2DG; an inducer of hypoglycaemia), deprivation of glucose, etoposide, staurosporine, UV irradiation, actinomycin D and cycloheximide [13]. Mitochondrial PHGPx also suppresses cell death caused by oxidized low-density lipoprotein, photosensitizers and cholesterol hydroperoxide in rabbit aortic smooth-muscle cells, human breast-cancer cells

Abbreviations used: AAPH, 2,2'-azobis-(2-amidinopropane) dihydrochloride; AIF, apoptosis-inducing factor; AM, acetoxymethyl ester; ANT, adenine nucleotide translocator; Atr, atractyloside; BA, bongkreikic acid; cGPx, cytosolic glutathione peroxidase; CL, cardiolipin; CLOH, hydroxycardiolipin; CLOOH, cardiolipin hydroperoxide; CsA, cyclosporin A; cyt *c*, cytochrome *c*; 2DG, 2-deoxyglucose; DOPC, dioleoyl phosphatidylcholine; LDH, lactate dehydrogenase; MCDP, 10-*N*-methyl-carbamoylmethylene; PA, phosphatidic acid; PC, phosphatidylcholine; PCOOH, phosphatidylcholine hydroperoxide; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PHGPx, phospholipid hydroperoxide glutathione peroxidase; PT, permeability transition; ROS, reactive oxygen species; t-BHP, t-butylhydroperoxide; VDAC, voltage-dependent anion channel.

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(MCF-7 cells) and lung breast-cancer cells respectively [17–19]. Inhibition of the release of cyt *c* by PHGPx suggests that lipid hydroperoxides might play a role in the release of cyt *c* from mitochondria. Cyt *c* binds loosely to inner membranes of mitochondria and has high affinity for cardiolipin (CL), which is a mitochondrion-specific phospholipid. CL loses its affinity for cyt *c* on peroxidation [14]. Our previous work suggests that peroxidation of CL might induce the dissociation of cyt *c* from mitochondrial inner membranes and that mitochondrial PHGPx might be involved in maintaining the association of cyt *c* with inner membranes by protecting CL from peroxidation [16].

CL, which is located exclusively in the inner membranes of mitochondria, plays a pivotal role as the boundary lipid of various proteins, such as NADH-ubiquinone oxidoreductase, cyt *c* oxidase, cyt *c* and the ANT, in the inner membranes of mitochondria [20,21]. The ADP/ATP translocase activity of ANT, which might be a key enzyme for the opening of PT pores, has an absolute requirement for CL [22]. It is not clear whether peroxidation of CL might be linked with the regulation of the opening of PT pores by ANT and the release of cyt *c* from outer membranes in a signalling pathway that leads to apoptotic cell death.

In the present study, we examined the effects of peroxidation of CL on the activity of ANT and the release of cyt *c* using reconstituted liposomes and transformed cells. We found that cardiolipin hydroperoxide (CLOOH) selectively decreased the translocase activity of ANT in reconstituted liposomes, in isolated mitochondria and in cells undergoing hypoglycaemia-induced apoptosis. Protection of CL from peroxidation by mitochondrial PHGPx prevented the loss of the activity of ANT, the opening PT pores and the release of cyt *c* in apoptosis.

EXPERIMENTAL

Materials

2DG, t-butylhydroperoxide (t-BHP), CsA and Atr were purchased from Sigma. Hen's-egg phosphatidylcholine (PC), phosphatidylethanolamine (PE) from *Escherichia coli*, bovine brain phosphatidylserine, porcine liver phosphatidylinositol, hen's-egg yolk phosphatidylglycerol (PG), hen's-egg lecithin phosphatidic acid (PA) and bovine brain sphingomyelin were also from Sigma. Bovine heart CL and BA were purchased from Funakoshi Co. (Tokyo, Japan), cyt *c* from horse heart and 2,2'-azobis-(2-amidinopropane)dihydrochloride (AAPH) were purchased from Sigma. Specific polyclonal antibodies against Bax and Bcl-2 were purchased from Transduction Laboratories (Lexington, KY, U.S.A.), similar antibodies against VDAC from Calbiochem–Novabiochem (La Jolla, CA, U.S.A.) and against cyt *c* and AIF from Pharmingen (San Diego, CA, U.S.A.). Calcein-acetoxymethyl ester (calcein-AM) was purchased from Dojindo Laboratories (Kumamoto, Japan). Other chemicals were obtained from Wako Pure Chemical Co. (Tokyo, Japan). Specific monoclonal antibodies against PHGPx were prepared as described previously [12].

Cell culture

We used our previously established lines of RBL2H3 cells that stably overexpress mitochondrial PHGPx (M15 cells) and the corresponding control cells (S1 cells), prepared by transfection with the expression vector without inserts [7]. Apoptosis in response to hypoglycaemia was induced by incubating cells with 100 mM 2DG in Dulbecco's modified Eagle's medium that

contained 5 % (v/v) foetal calf serum, as described previously [13].

Assessment of cell viability and release of cyt *c*

S1 and M15 cells were plated at 0.5×10^5 cells/well in flat-bottomed 96-well-culture plates and cultured for 24 h. Apoptotic cell death was induced by treating cells with indicated doses of 2DG for 16 h. Cells were incubated for 10 min with the indicated concentrations of CsA and BA to induce closure of PT pores before exposure to 2DG. Then they were incubated for 16 h with 100 mM 2DG. Quantification of released lactate dehydrogenase (LDH) was used to determine cell viability, as described previously [13]. For analysis of the release of cyt *c* and AIF from mitochondria into the cytosol, proteins in supernatants from digitonin-treated cells were precipitated by the addition of trichloroacetic acid and separated by SDS/PAGE, as described elsewhere [16]. Bands of proteins were transferred on to a PVDF membrane (Millipore, Bedford, MA, U.S.A.) as described previously [16]. Cyt *c* and AIF were detected with an enhanced chemiluminescence Western-blotting analysis system (Amersham, Buckinghamshire, U.K.) with monoclonal antibodies specific for cyt *c* and AIF. Apoptotic cell death was also evaluated by confocal fluorescence microscopy after staining with Hoechst 33258 and anti-cyt *c* antibodies. Hoechst staining was performed with minor modification as described previously [13]. Immunohistochemistry of cyt *c* was also performed as described previously [7]. The cells were analysed with a Zeiss Lasershape LSM510 scanning confocal microscopy system (Zeiss LSM510, Oberkochen, Germany).

Isolation of mitochondria

Mitochondria were isolated from S1 and M15 cells as described previously [16]. In brief, 5×10^7 cells were scraped from plates and resuspended in 2 ml of buffer A [0.25 M sucrose, 1 mM EDTA, 3 mM imidazole and 0.1 % (v/v) ethanol (pH 7.2) with 10 µg/ml leupeptin, 10 µg/ml antipain, 10 µg/ml chymostatin, 10 µg/ml pepstatin and 100 µg/ml PMSF]. The cells were then lysed mechanically at 4 °C in a cylindrical homogenizer. Lysates were centrifuged for 10 min at 750 *g*, and then supernatants were centrifuged at 10 000 *g* to pellet the mitochondrial fraction. The purity of isolated mitochondria was determined according to our previous studies [7,10]. Cyt *c* oxidase, NADPH-cyt *c* reductase and LDH were distributed 75.6, 16.7 and 2.5 % respectively in mitochondrial fractions.

Release of cyt *c* from isolated mitochondria

Mitochondria were isolated from S1 and M15 cells as described above. The isolated mitochondria were suspended at 0.5 mg of protein/ml in mitochondrion-isolation buffer [210 mM mannitol, 70 mM sucrose, 3 mM Hepes/KOH (pH 7.4), 0.2 mM EGTA and 0.5 mg/ml BSA]. Release of cyt *c* was monitored after adding the indicated doses of t-BHP and Atr with or without prior treatment with CsA and BA at the indicated concentration. After incubation for 30 min at 37 °C, mitochondria were centrifuged at 10 000 *g* for recovery of supernatants that contained released cyt *c*. Aliquots of supernatants were fractionated by SDS/PAGE, bands of protein were transferred on to a PVDF membrane and cyt *c* was detected as described above. The measurements of lipid peroxidation were monitored after the addition of 200 µM t-BHP. After incubation for 30 min at 37 °C, total lipids of mitochondria were extracted by the method of Bligh

and Dyer [23]. Lipid hydroperoxide was quantified by the 10-*N*-methyl-carbamoylmethylene (MCDP) method [24] using phosphatidylcholine hydroperoxide (PCOOH) as the internal standard [16]. The lipid extract was dried under a stream of nitrogen gas, and the residue was dissolved in 0.1 ml of 2-propanol. Then, 1 ml of MCDP reagent, containing 40 mM MCDP, 67.5 mg/ml bovine haemoglobin and 0.1 % Triton X-100 in 0.1 M Mops (pH 6.0), was added to the lipid extract and incubated at 30 °C for 20 min. Lipid hydroperoxide was quantified by measuring the absorbance at 675 nm as described previously [24]. Lipid hydroperoxide was separated from the added t-BHP, since t-BHP was distributed to the water phase, and not to the organic phase, by the method of Bligh and Dyer [23].

Assessment of the opening of mitochondrial PT pores

Mitochondria (1 mg of protein/ml) isolated from cultured cells were suspended in 0.25 M sucrose, 10 mM Tris/Mops (pH 7.4), 5 mM succinate, 5 mM rotenone, 1 mM KH_2PO_4 and 10 mM EDTA. These mitochondria were incubated with or without 1 μM CsA for 1 min. Then a swelling was induced by 100 μM t-BHP and the extent of swelling was estimated using the decrease in the absorbance value at 540 nm in a spectrometer (F-4500; Hitachi, Tokyo, Japan). To measure the release of calcein from PT pores, we suspended isolated mitochondria at 0.5 mg of protein/ml in a mitochondrion-isolation buffer. Calcein was introduced into the mitochondrial matrix by incubation with 1 μM calcein-AM, which is capable of permeating mitochondrial membranes. The mitochondria were then washed and incubated with or without 1 μM CsA for 5 min before incubation with 100 μM t-BHP. Fluorescence was measured at 530 nm with CytoFluor™ plate reader (model 4000; PerSeptive Biosystems, Framingham, MA, U.S.A.) with excitation at 485 nm.

Fusion of liposomes with mitochondrial inner membranes

Liposomes (small unilamellar vesicles) were prepared by sonicating 1.7 mg of phospholipids in 1 ml of 25 mM phosphate buffer (pH 6.7) with the microtip probe of a Branson sonicator (model 250) at 40 W for six cycles of 2 min in an ice bath. After centrifugation of liposomes at 10 000 *g* for 20 min, the supernatant was used for experiments. Liposomes were fused with mitochondrial membranes essentially as described by Hackenbrock and Chazotte [25] and Paradies et al. [26]. In brief, 20 ml of the preparation of freshly sonicated liposomes was added to 0.5 mg of mitochondrial proteins in mitochondrion-isolation buffer, at room temperature (25 °C), with constant stirring. After a 40 min incubation, the mixture was centrifuged at 10 000 *g* for 20 min, and the mitochondrial pellet was washed and resuspended in ADP-import buffer (250 mM sucrose/20 mM Hepes/10 mM KCl/5 mM succinate/3 mM KHO_4 /1.5 mM MgCl_2 /1 mM EGTA/5 mM rotenone; pH 7.2).

Assay of ANT-dependent import of ADP

Pellets of isolated mitochondria treated with or without t-BHP, or fused with liposomes, or fractionated from apoptotic cells treated with 100 mM 2DG were resuspended in 900 ml of ADP-import buffer as described above. Atr, at a final concentration of 50 μM , was added to 400 μl of the mitochondrial suspension before the addition of 0.3 μCi (5.3 nmol) of [^{14}C]ADP (New England Nuclear, Boston, MA, U.S.A.). At the same time, [^{14}C]ADP was

added similarly to 400 μl of mitochondrial suspension without Atr. Both samples were incubated for 1 min on ice before the addition of 700 μl of ADP-import buffer that contained 50 μM Atr to stop the reaction. Samples were washed twice in ADP-import buffer, mixed with liquid-scintillation cocktail and the radioactivity due to [^{14}C]ADP was determined. The ANT-dependent uptake of ADP was calculated as the difference in radioactivity between the sample pretreated with Atr and the untreated sample.

Purification of ANT and its incorporation into liposomes

We purified ANT from rat brain mitochondria as described previously with slight modifications [27]. Brain mitochondria were prepared from brains of 5 rats by homogenization in a blender and differential centrifugation in a medium that contained 220 mM mannitol, 70 mM sucrose, 10 mM Hepes, 0.2 mM EDTA and 1 mM 2-mercaptoethanol (pH 7.4). Isolated brain mitochondria (20 mg of protein) in 1 ml of isolation medium were incubated for 5 min with an equal volume of extraction buffer [40 mM KH_2PO_4 /40 mM KCl/2 mM EDTA/6 % (v/v) Triton X-100; pH 6.0]. The suspension was centrifuged for 30 min at 24 000 *g* and the supernatant was loaded on to a column that contained 1 g of dry hydroxyapatite (Bio-Rad Laboratories). The column was eluted with extraction buffer and the flow-through fraction was collected and diluted with an equal volume of column buffer (20 mM Mes/0.2 mM EDTA/0.5 % Triton X-100; pH 6.0). This sample was applied on to a 1 ml HiTrapSP-cation exchanger column (Pharmacia, Piscataway, NJ, U.S.A.), connected to an HPLC system and eluted with a gradient of 0–1 M NaCl in column buffer. The resultant fractions were analysed by SDS/PAGE [12 % (w/v) polyacrylamide]. Immuno-detection was performed with polyclonal antibodies specific to a peptide derived from ANT, namely, CLDFARTRLAADV GK prepared as described elsewhere [6].

ANT was purified to a single band of protein and was incorporated into dioleoyl phosphatidylcholine (DOPC) vesicles that contained various phospholipids (10 %, w/w). The fractions containing ANT were mixed with the liposomes and the mixture was dialysed overnight against 125 mM sucrose and 10 mM Hepes (pH 7.4). The vesicles were loaded with 500 mM ATP and 10 mM KCl by sonication and unincorporated ATP was removed by chromatography on Sephadex G50 (Pharmacia) in 125 mM sucrose and 10 mM Hepes. After chromatography, the permeability to ATP was examined by centrifugation for 30 min at 400 000 *g* and the ATP in the supernatant and pellet was quantified by luciferine–luciferase assay as described previously [13].

Quantification of ADP/ATP translocase activity in liposomes prepared with ANT

Liposomes loaded with ATP and KCl and containing purified ANT were suspended in 125 mM sucrose and 10 mM Hepes (pH 7.4). The vesicles were incubated for 30 min at room temperature with 100 μM ADP and analysed for the ATP released. After incubation, the vesicles were centrifuged as described above and the liberated ATP in the supernatant was quantified by a luciferine–luciferase assay as described previously [13]. Luminescence was monitored over a 10 s period with a CytoFluor™ plate reader. The release of ATP was also measured in the presence of Atr at a concentration of 1 mM. The CLOOH in the liposomes that contained purified ANT was reduced by incubation with PHGPx purified from rat testis mitochondria as described previously [16], for 10 min at 37 °C with 0.5 mM glutathione

Figure 1 Expression of proteins present in PT pores of mitochondria

Proteins from mitochondria isolated from control cells (S1) and cells that overexpress mitochondrial PHGPx (M15) were separated by SDS/PAGE. Each protein was detected by immunoblotting analysis with appropriate antibodies.

before measurement of ANT activity. In some experiments, we treated liposomes that contained ANT with AAPH, H_2O_2 and t-BHP at indicated concentrations, with or without 100 mM $FeSO_4$ or 1 $\mu g/ml$ of cyt *c* for 30 min. Treated vesicles were loaded with 500 mM ATP and 10 mM KCl by sonication and unincorporated ATP was subsequently removed by chromatography on Sephadex G50. After chromatography, the release of ATP on addition of ADP was monitored by the luciferine–luciferase assay as described above.

Preparation of CLOOH and hydroxycardiophilin (CLOH)

Thin films of bovine heart CL were allowed to autoxidize at 37 °C overnight. CLOOH was isolated by HPLC with UV detection as described previously [16]. In brief, oxidized CL, dissolved in a mixture of ethanol and hexane (1:4, v/v), was injected into an HPLC system (model 10A; Shimadzu, Tokyo, Japan) with a silica gel column (LiChrosorb Si60; Merck, Darmstadt, Germany) at a flow rate of 2 ml/min. Elution was performed with solvent A, which was a mixture of hexane, ethanol and water (360:60:1, by vol.) and solvent B, a mixture of hexane, ethanol and water (225:150:7, by vol.) as follows: 0–20% B in A for 20 min; 20% B in A for 10 min; 20–95% B in A for 5 min; 95% B in A for 5 min; 95–0% B for 5 min; with final equilibration of the column in 100% A for 15 min. Phospholipids were monitored, at 205 and 235 nm, with a UV detector (SPD-10A; Shimadzu). The lipid fractions that contained CLOOH were collected and stored on ice. They were evaporated to dryness under a stream of nitrogen and the residue was dissolved in chloroform. Total hydroperoxide was quantified by the MCDP method [24] with PCOOH, prepared as described previously [16], as the standard. CLOH was prepared by reducing purified CLOOH with $NaBH_4$. PCOOH was prepared by photooxidation of hen's-egg yolk PC as described previously [16].

RESULTS

Inhibition of the opening of PT pores in isolated mitochondria that overexpress PHGPx

We examined the expression of proteins that are included in PT pores in isolated mitochondria from control cells (S1 cells) and cells that overexpressed mitochondrial PHGPx (M15 cells) by immunoblotting analysis (Figure 1). There were no significant differences between S1 and M15 cells in terms of the respective levels of expression in mitochondria of ANT, VDAC, Bcl-2, Bax and cyt *c*. The amount of PHGPx was significantly enhanced

Figure 2 Prevention of t-BHP-induced opening of PT pores by mitochondrial PHGPx in isolated mitochondria

(A, B) Prevention of the t-BHP-induced swelling of mitochondria by mitochondrial PHGPx. Mitochondria (1 mg/ml), isolated from S1 cells (A) and M15 cells (B), were incubated with (■, ▲) or without (●) 100 μM t-BHP with (■, ▲) or without (●) prior treatment using 1 μM CsA. Then light scattering due to swelling of mitochondria was monitored at 540 nm. (C, D) Prevention of the t-BHP-induced release of calcein by mitochondrial PHGPx. Mitochondria (0.5 mg/ml), isolated from S1 cells (C) and M15 cells (D), were incubated for 15 min with calcein-AM. The mitochondria were washed and then either left untreated (▲, ●) or treated (■) for 5 min with 5 μM CsA. The mitochondria were then exposed to 100 μM t-BHP (■, ▲) or not exposed (●). Aliquots of mitochondria were then removed and the fluorescence of calcein in mitochondria was measured at the indicated time periods as described in the text.

in isolated mitochondria from M15 cells. The purity of isolated mitochondria was determined as described in our previous papers [7,10].

The opening of PT pores is associated with expansion of the space occupied by the mitochondrial matrix, namely, swelling of the matrix. t-BHP induced the swelling of mitochondria isolated from control cells (S1 cells), as assessed in terms of the decrease in the absorbance at 540 nm (Figure 2A). Mitochondrial swelling in response to t-BHP was significantly suppressed by CsA, a specific inhibitor of the opening PT pores. Strong inhibition of swelling was also observed for t-BHP-treated mitochondria isolated from cells that overexpressed mitochondrial PHGPx (M15 cells; Figure 2B). Figures 2(C) and 2(D) show the release of calcein from isolated mitochondria after exposure to t-BHP. Mitochondria were preloaded with calcein, which gets trapped in the mitochondrial matrix after hydrolytic removal of the AM group of calcein-AM by non-specific esterases. On exposure of the mitochondria to t-BHP, the fluorescence due to calcein was promptly lost from the mitochondria as a result of the opening of PT pores (Figure 2C). The release of calcein from isolated mitochondria in response to t-BHP was not observed in mitochondria that overexpressed PHGPx (Figure 2D).

Figure 3 Effects of mitochondrial PHGPx on the t-BHP and Atr-induced release of cyt *c* from isolated mitochondria

Prevention of the t-BHP-induced (A, B) and Atr-induced (C, D) release of cyt *c* by mitochondrial PHGPx. Mitochondria (0.5 mg/ml) were isolated from cells that overexpressed mitochondrial PHGPx (M15 cells; B, D) and from control cells (S1 cells; A, C). Isolated mitochondria were incubated with the indicated concentrations of t-BHP or 6 μ M Atr for 30 min with or without prior treatment with CsA or BA, as indicated. After incubation, samples were centrifuged and amounts of released cyt *c* in supernatants were determined by Western blotting with specific antibodies. Mt, mitochondrial fraction.

Treatment with t-BHP resulted in the dose-dependent liberation of cyt *c* from mitochondria isolated from S1 cells (Figure 3A). The liberation of cyt *c* was blocked in the presence of CsA or BA which binds ANT and closes the PT pores. By contrast, Atr which can induce the opening of PT pores initiated the release of cyt *c* from mitochondria isolated from S1 cells (Figure 3C). The Atr-induced release of cyt *c* was inhibited by prior treatment of the isolated mitochondria with CsA or BA (Figure 3C). By contrast, no t-BHP-induced release of cyt *c* was observed in

mitochondria isolated from M15 cells (Figure 3B). However, the release of cyt *c* from mitochondria in M15 cells was observed on treatment with Atr (Figure 3D) and the Atr-induced release of cyt *c* from isolated mitochondria in M15 cells was blocked in the presence of CsA or BA.

Mitochondrial PHGPx suppresses the loss of the ADP/ATP translocase activity of ANT induced by exposure of mitochondria to t-BHP or exogenous CLOOH

The ADP/ATP translocase activities of ANT in isolated mitochondria from S1 and M15 cells were 2.72 ± 0.43 and 2.80 ± 0.52 nmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ respectively. No significant changes in ANT activity were observed between S1 and M15 cells. Treatment of isolated mitochondria from S1 cells with t-BHP reduced the activity of ANT to 30% of the control value (Figure 4A). By contrast, a much smaller decrease in the activity of ANT was observed in t-BHP-treated mitochondria from M15 cells.

The generation of lipid hydroperoxide was monitored in mitochondria isolated from S1 and M15 cells after the exposure of isolated mitochondria to t-BHP for 30 min. Total lipids of isolated mitochondria were extracted and separated from t-BHP in the water fraction. Lipid hydroperoxide was detected by the methylene blue (MCDP) method [24]. An apparent production of lipid hydroperoxide was observed in the t-BHP-treated mitochondria prepared from S1 cells (Figure 4B), whereas the formation of lipid hydroperoxide was effectively suppressed in the t-BHP-treated mitochondria prepared from M15 cells where the mitochondrial PHGPx was overexpressed (Figure 4B). No difference in the decrease of hydroperoxide was observed between the water fractions of t-BHP-treated isolated mitochondria of S1 and M15 cells (results not shown). These results indicated that mitochondrial PHGPx would have prevented the inhibition of the activity of ANT by suppression of t-BHP-induced peroxidation of mitochondrial lipid.

We next examined the effects of lipid hydroperoxides on the activity of ANT by fusing liposomes with isolated mitochondria.

Figure 4 Effects of addition of t-BHP and various exogenous phospholipid hydroperoxides on the translocase activity of ANT in isolated mitochondria

(A) Suppression of the t-BHP-induced decrease in the activity of the ANT in isolated mitochondria by mitochondrial PHGPx. The loss of ANT activity after treatment with t-BHP was assessed in terms of the uptake of [14 C]ADP by mitochondria isolated from M15 and S1 cells. The ANT-dependent uptake of ADP by isolated mitochondria treated with 100 μ M t-BHP is expressed, as a percentage, relative to the uptake by isolated mitochondria not treated with t-BHP. (B) Suppression of the increase in lipid hydroperoxide in t-BHP-treated isolated mitochondria by mitochondrial PHGPx. The lipid fraction was extracted by the method of Bligh and Dyer [23] after each mitochondria isolated from S1 cells (black bar) and M15 cells (hatched bar) was treated with or without 200 μ M t-BHP for 30 min. The amount of lipid hydroperoxide was quantified by the methylene blue (MCDP) method as described in the Experimental section. (C) Decrease in ANT activity in the product of the fusion of isolated mitochondria with liposomes that contained CLOOH. Mitochondria isolated from M15 and S1 cells were fused with liposomes prepared with various phospholipids, namely, PC + CL, PC + CLOOH, PC alone and PC + PCOOH (molar ratio was 1 : 1 for each mixture). The ANT-dependent uptake of [14 C]ADP by mitochondria fused with liposomes is expressed, as a percentage, relative to that by similar mitochondria not fused with liposomes. Results are expressed as means $\pm$ S.D. from five independent experiments.

Fusion was performed basically as described by Hackenbrock and Chazotte [25] and Paradies et al. [26] to allow incorporation of various phospholipids into mitochondrial inner membranes. A significant reduction of the activity of ANT was not found after fusion of liposomes that consisted of non-oxidized phospholipids, such as CL or PC, with mitochondria from S1 cells (Figure 4C). On the other hand, the activity of ANT decreased significantly to 20% of the control level on fusion of liposomes that contained CLOOH with these mitochondria. No significant decrease in the activity of ANT was observed when mitochondria were fused with liposomes prepared with PCOOH. However, CLOOH in liposomes failed to reduce the activity of ANT when liposomes were fused with mitochondria from M15 cells, indicating that mitochondrial PHGPx effectively prevented the decrease in the activity of ANT by CLOOH incorporated by fusion into mitochondrial inner membranes.

Inhibition of the activity of ANT by CLOOH in liposomes prepared with ANT

Liposomes were prepared with purified ANT from rat brain to allow evaluation of the effects of CLOOH on ANT activity in a simpler system. We incorporated ANT into liposomes composed of 90% (mol/mol) DOPC with the balance made up of several classes of phospholipids, which we examined in terms of requirements for the activities of ANT (Figure 5A). The translocase activity of ANT was supported by CL or PG, but not by other anionic phospholipids such as PA, phosphatidylserine, phosphatidylinositol and PE. Maximum translocase activity of ANT was observed when liposomes contained 10% (mol/mol) CL (Figure 5B). No further enhancement of the activity of ANT was observed when liposomes were prepared with 20% CL (results not shown).

CLOOH did not support the expression of the activity of ANT. However, the reduced form of CLOOH, namely CLOH, enhanced the activity of ANT similarly to CL (Figure 6A). The failure of CLOOH to activate ANT was not due to the destruction of liposome membranes, because no leakage of entrapped ATP was observed from liposomes composed exclusively of 10% (mol/mol) CLOOH and 90% DOPC (results not shown). The activity of ANT in liposomes prepared with CLOOH and DOPC was similar to that of liposomes prepared with CLOH and DOPC on treatment of the former liposomes with PHGPx.

The activity of ANT incorporated into liposomes prepared with PC and CL was reduced by the inclusion of CLOOH in the liposomes and the effect of CLOOH was dose-dependent (Figure 6B). The activity of ANT was similar to the control activity when the same amounts of CLOOH (10%) and CL were used. By contrast, PCOOH had no similar inhibitory effect on the activity of ANT (Figure 6B).

Exogenously added water-soluble hydroperoxides, such as H₂O₂ and t-BHP, did not affect the activity of ANT, whereas AAPH, as a lipid-soluble radical initiator, caused significant loss of ANT activity (Figure 7A). We also observed inhibition of ANT activity by H₂O₂ and t-BHP on addition of FeSO₄ or cyt *c*, both capable of inducing iron-catalysed peroxidation of lipids (Figure 7B).

Protection from inactivation of ANT in cells that overexpress PHGPx during hypoglycaemia-induced apoptosis

S1 cells were induced to undergo apoptotic cell death by exposure to 2DG, which induces hypoglycaemia, whereas M15 cells were resistant to these apoptotic effects of 2DG as shown

Figure 5 The translocase activity of ANT in liposomes is specifically dependent on CL

The specificity of phospholipid dependence (A) and CL dependence (B) of the translocase activity of ANT ANT-reconstituted in liposomes. To measure the activity of ANT, ANT-reconstituted liposomes containing ATP were treated with 400 μ M ADP for 30 min. The ADP-induced release of ATP was then quantified by a luciferase–luciferase assay. (A) ANT was purified from rat brain and incorporated into liposomes prepared with DOPC and 10% of the indicated phospholipid as described in the text. Results are expressed as means $\pm$ S.D. from three independent experiments. PI, phosphatidylinositol; PS, phosphatidylserine. (B) The level of CL in liposomes was 10% (■), 2% (▲) and 0% (●) relative to the level of DOPC (mol/mol) and all liposomes contained purified ANT. Results are typical of three independent experiments that yielded similar results.

previously [13,16]. In the present study, we examined intracellular localization of cyt *c* and nuclear condensation during 2DG-induced apoptosis using a confocal fluorescence microscopy. Hypoglycaemia-induced apoptosis was strongly suppressed by pretreatment of cells with CsA and BA. The effects of CsA and BA were dose-dependent (Figures 8A and 8B). Cyt *c* was distributed in mitochondria of untreated S1 cells (Figure 9A) and M15 cells (Figure 9E), because the profile of red fluorescence of Cy3-conjugated anti-cyt *c* were identical with that of mitochondrial cyt *c* oxidase (results not shown). The release of cyt *c* from mitochondria to the cytosol and nuclear condensation were observed in S1 cells treated with 2DG (Figure 9B), but not in M15 cells treated with 2DG (Figure 9F). These results confirmed that mitochondrial PHGPx suppresses the release of cyt *c* from mitochondria to cytosol and apoptotic cell death after exposure to 2DG. Hypoglycaemia-induced apoptosis was strongly suppressed by pretreatment of cells with CsA (Figure 9D) and BA (Figure 9C). The release of cyt *c* and AIF from mitochondria of cells treated with 2DG was completely suppressed by prior treatment with CsA or BA (Figures 8C, 9C and 9D), indicating that the opening of PT pores might be involved in the release of proapoptotic factors and the initiation of 2DG-induced apoptosis.

Figure 6 Effects of CLOOH on the translocase activity of ANT in reconstituted liposomes

To measure the translocase activity of ANT, we treated ANT-reconstituted liposomes containing ATP with 400 μ M ADP for 30 min. The ADP-induced release of ATP was then quantified by a luciferine–luciferase assay. **(A)** Effects of oxidized CL on the ANT activity of liposomes. Purified ANT was incorporated into liposomes containing 90% DOPC and 10% the indicated form of CL. Aliquots of liposomes containing 10% CLOOH and ANT were incubated with or without purified PHGPx for 10 min with 0.5 mM glutathione before measurement of ANT activity. **(B)** Competitive CLOOH inhibition of the translocase activity of ANT in liposomes. Purified ANT was incorporated into liposomes containing 10% CL and the indicated relative levels (%) of CLOOH or PCOOH. Results are expressed as means $\pm$ S.D. from three independent experiments.

We estimated the activity of ANT in S1 and M15 cells after their exposure to 2DG. The activity of ANT was unaffected for 2 h, but decreased dramatically to 50% of the control level at 4 h in S1 cells (Figure 10A). However, minimal inactivation of ANT was observed in M15 cells during an 8 h incubation (Figure 10B). In parallel, cyt *c* and AIF were released from mitochondria of S1 cells at 4 h (Figure 10C), whereas no similar liberation of proapoptotic factors was observed in M15 cells (Figure 10D).

DISCUSSION

The release of cyt *c* from mitochondria can be induced by various pro-oxidant agents, such as tumour necrosis factor α [28], ceramide [29], glutamate [30], amyloid β -peptide [31], as well as by glucose depletion [13] and withdrawal of growth factors

Figure 7 Effects of soluble hydroperoxides and AAPH on the translocase activity of ANT in reconstituted liposomes

(A) Effect of soluble hydroperoxides and AAPH on the activity of purified ANT incorporated into liposomes containing 10% CL and 90% DOPC. The liposomes were loaded with ATP and treated with AAPH, H₂O₂ or t-BHP at the indicated concentrations for 30 min. Then they were treated with 400 μ M ADP for 30 min. The ADP-induced release of ATP was quantified by means of the luciferine–luciferase assay. **(B)** Effects of incubation with soluble hydroperoxides and Fe²⁺ or cyt *c* on the translocase activity of ANT in liposomes. Purified ANT was incorporated into liposomes containing 10% CL and 90% DOPC and the liposomes were loaded with ATP. The liposomes were then incubated with or without 200 mM FeSO₄ or 10 μ g/ml of cyt *c* in addition to 0.4 mM H₂O₂ or t-BHP for 30 min. Finally, they were incubated with 400 μ M ADP for 30 min. The ADP-induced release of ATP was then quantified by a luciferine–luciferase assay. Results are expressed as means $\pm$ S.D. from three independent experiments.

[32]. The involvement of oxidative stress in the initiation of apoptosis has been widely discussed. The opening of PT pores is a prerequisite for the release of cyt *c* and AIF from mitochondria. The opening of such pores can be induced by extramitochondrial and intramitochondrial reactive oxygen species (ROS), such as t-BHP and H₂O₂. The opening of PT pores induced by pro-oxidants can be prevented by the exogenous addition of catalase and by any thiol-specific antioxidant that can reduce H₂O₂ directly [33]. Oxidative stress also induces the loss of activity of ANT in isolated mitochondria [34]. The mechanisms that regulate the opening of PT pores in response to ANT and the relationship between the oxidative loss of ANT activity and the release of cyt *c* remain to be characterized.

In the present study, using isolated mitochondria, we found that overexpression of PHGPx in mitochondria suppressed the

Figure 8 Effects of CsA and BA on 2DG-induced cell death and the release of cyt *c* and AIF

After treatment for 30 min with the indicated doses of BA (A) and CsA (B), S1 cells (●) and M15 cells (□) were incubated with 100 mM 2DG for 16 h. (A, B) Cell viability was examined in terms of the release of LDH during a 16 h period after the addition of 2DG. (C) The release of cyt *c* and AIF was detected in the cytosol of S1 cells treated with 2DG for 6 h by immunoblotting analysis with appropriate antibodies. Results are expressed as means $\pm$ S.D. from three replicate experiments in each case.

inactivation of ANT, with resultant inhibition of the opening of PT pores and the release of cyt *c* from isolated mitochondria treated with t-BHP (Figures 2–4). In our previous study [7], we showed that the specific activity of PHGPx in isolated mitochondria from M15 cells was 1.8 times higher than that in S1 cells, although no significant changes of cytosolic glutathione peroxidase (cGPx) activities in mitochondria were found between isolated mitochondria from S1 and M15 cells. cGPx reduces t-BHP and H_2O_2 effectively but does not reduce phospholipid hydroperoxide directly, but not reduce t-BHP effectively, because the k_1 value for phospholipid hydroperoxide of PHGPx was 70 times higher than that of cGPx, but the k_1 value for t-BHP of PHGPx was 60 times lower than that of cGPx as reported previously [35]. In t-BHP-treated isolated mitochondria, mitochondrial PHGPx effectively suppressed the production of mitochondrial lipid hydroperoxide (Figure 4B). These results indicated that inactivation of ANT was dependent on peroxidation of mitochondrial lipid. Studies of variously constituted liposomes revealed that PHGPx protected the activity of ANT from the negative effects of CLOOH. In particular, ANT activity recovered on conversion of CLOOH into CLOH by PHGPx (Figure 6A). However, mitochondrial PHGPx failed to block the Atr-induced opening of PT pores and release of cyt *c*, suggesting that lipid peroxides might not contribute to the opening of PT pores caused by direct binding of Atr to ANT.

Figure 9 Pretreatment of CsA and BA suppressed 2DG-induced release of cyt *c* and nuclear condensation

S1 (A, B) and M15 cells (E, F) were incubated with (B, F) or without (A, E) 100 mM 2DG for 16 h. After treatment for 30 min with 10 μM BA (C) or 10 μM CsA (D), S1 cells (C, D) were incubated with 100 mM 2DG for 16 h. Cells were fixed, washed with PBS and then stained with Hoechst 33258 (blue) and immunostained with anti-cyt *c* antibody (red).

Hypoglycaemia caused by 2DG in S1 cells induced a rapid depletion of intracellular ATP, production of CLOOH and ROS within 2 h, the release of cyt *c* from mitochondria at 4 h and the induction of cell death after 10 h [13]. We observed significant loss of ANT activity within 4 h of exposure to 2DG in S1 cells (Figure 10A). The production of CLOOH, before the decrease in ANT activity in cells that are undergoing apoptosis in response to hypoglycaemia, might be of interest, because our studies with liposomes showed that CLOOH reduced the activity of ANT to the basal level. Inactivation of ANT was protected by the suppression of the peroxidation of CL via overexpression of mitochondrial PHGPx with resultant inhibition of the release of cyt *c* and AIF in hypoglycaemia-induced apoptosis (Figures 9F, 10B and 10D). These results suggest that peroxidation of CL might be tightly linked with the regulation of ANT activity, opening of PT pores and release of cyt *c* from mitochondria. These results also indicate that mitochondrial PHGPx might be involved in the modulation of the activity of ANT and opening of PT pores via modulation of levels of CLOOH in the mitochondria. Vander Heiden et al. [36] reported that loss of ANT activity during apoptosis was induced by withdrawal of interleukin 3. They showed that withdrawal of interleukin 3 decreased the rates of glucose metabolism and ATP production [37]. Hockenbery et al. [38] showed that apoptosis induced by the withdrawal of interleukin 3 is caused by production of H_2O_2 . The molecular mechanism responsible for the loss of ANT activity is unclear. However, peroxidation of CL might be one of the events responsible for the decrease in the activity of ANT.

Figure 10 Mitochondrial PHGPx suppresses the loss of the translocase activity of ANT and the release of cyt *c* and AIF during 2DG-induced apoptosis

S1 (A, C) and M15 cells (B, D) were treated with 2DG for the indicated time periods. (A, B) Mitochondria were isolated from S1 (A) and M15 (B) cells exposed to 2DG. The ANT-dependent uptake of ADP by the mitochondria was assessed by incubating them in the presence of [14 C]ADP with or without a prior incubation with Atr. The mean ANT-dependent uptake of ADP by mitochondria from 2DG-treated cells is shown as a percentage of that by mitochondria from non-treated cells. Results are expressed as means $\pm$ S.D. from three independent experiments. (C, D) For the detection of released cyt *c* and AIF from mitochondria, cytosolic fractions were collected after treatment of cells with 10 μ g/ml of digitonin for 10 min. The release of cyt *c* and AIF was detected by immunoblotting analysis with appropriate antibodies.

We obtained more direct evidence for the inhibition of ANT activity by CLOOH in studies of liposomes prepared with ANT and liposomes fused with mitochondrial inner membranes (Figures 4C and 6–8). We used a previously reported method for fusion of lipid vesicles with mitochondrial membranes to increase relative levels of various phospholipids in mitochondrial inner membranes [25,26,39–42]. Using this approach, we showed that there was a decrease in the activity of ANT when liposomes prepared with CLOOH were fused with isolated mitochondria. However, such inhibitory effect was not found with PCOOH (Figure 4C). In liposomes prepared with ANT, inclusion of CLOOH instead of CL did not enhance the activity of ANT. In contrast, reduced CLOOH (CLOH) could play the role of CL (Figure 6). The activity of ANT was also supported by PG but not by PA (Figure 5A). These results indicate that ANT recognizes the structure of PG and the hydrophobic moiety of esterified fatty acid. CLOOH competitively inhibited the CL-dependent activity of ANT in liposomes prepared with ANT, but PCOOH did not, indicating that CLOOH displaced CL from a specific-binding site in ANT. Although, the oxidation of mitochondrial membrane lipids is not specific to CL during apoptotic cell death, ANT was inactivated only by CLOOH and not by PCOOH, suggesting that ANT might sense levels of CLOOH in the mitochondrial membrane that provide a signal for apoptotic death and release of cyt *c*.

The mechanism of opening of CLOOH-induced PT pores via modification of ANT remains to be resolved. Purified ANT

from bovine heart contains six molecules of tightly bound CL/protein dimer [43]. CL is strongly immobilized on the surface of ANT, with extensive broadening of its [31 P]NMR signal, a property that is very unusual for protein-bound phospholipids [43]. Dissociation of CL from ANT can be achieved only under denaturing conditions and results in complete loss of ANT function [44]. Treatment of phospholipase A₂ with a detergent-solubilized ANT–CL complex also induces a conformational change in the ANT, indicating that intact CL including esterified fatty acid is required for the appropriate conformation of ANT [44]. Brustovetsky and Klingenberg [45] showed that the bivalent cation Ca²⁺ interferes with the interaction of ANT and CL, with a resultant structural change in ANT. In the study of ANT-containing liposomes, CLOOH reversibly and competitively inhibited the activity of ANT (Figure 6). Our results suggest that CLOOH might alter the activity and conformation of ANT for opening of PT pores and release of cyt *c*.

CL interacts with a large number of mitochondrial proteins, such as ANT, cyt *c* complexes I, III and IV and F₀F₁-ATPase in the inner membrane. CL is required for the folding and activity of these mitochondrial enzymes [46–48]. Peroxidation of CL induces a decrease in the activity of complexes I and IV, which might induce the loss of mitochondrial membrane potential, a common phenomenon in apoptosis [41,49]. Peroxidation of CL induces the dissociation of cyt *c* from the mitochondrial inner membrane [16]. Ostrander et al. [50] demonstrated that a decrease in the synthesis of CL is associated with the release of cyt *c* in the palmitate-induced apoptosis of cardiomyocytes. Grijalba et al. [51] showed that Ca²⁺-dependent packing of CL and formation of CL domains in mitochondria induced the generation of ROS by the respiratory chain. Lutter et al. [52] also showed that CL is required for specific targeting of tBid to mitochondria. All these previous studies and also the present one suggest that modification of CL, for example, by oxidation, might be a critical event in the regulation of apoptotic signalling, such as the release of mitochondrial apoptotic factors.

Mitochondrial PHGPx suppresses apoptosis that occurs via the mitochondrial-death pathway and involves the peroxidation of CL [13,16]. We showed previously that mitochondrial PHGPx inhibits the release of cyt *c* from mitochondrial inner membranes by suppressing the peroxidation of CL in apoptosis. Mitochondrial PHGPx is located in the intermembrane space, in particular, at a contact site that was located in the PT pore complex, which includes ANT [53]. The present study demonstrates that mitochondrial PHGPx plays an important role in regulating the activity of ANT and the opening of PT pores. Mitochondrial PHGPx is synthesized at significantly elevated levels in late spermatocytes during human spermatogenesis [12]. Apoptosis might be involved at various stages of spermatogenesis and might regulate both development and quality control during the production of spermatozoa. We reported recently that failed induction of the expression of mitochondrial PHGPx as an anti-apoptotic factor might have a major effect on spermatogenesis and might induce a significant decrease in the production of mature spermatozoa with resultant infertility [12]. Thus mitochondrial PHGPx might play a physiologically important role as a natural anti-apoptotic factor.

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