Mitochondrial permeability transition induced by reactive oxygen species is independent of cholesterol-regulated membrane fluidity

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Abstract Cholesterol enrichment of rat liver mitochondria (CHM) impairs atractyloside-induced mitochondrial permeability transition (MPT) due to decreased membrane fluidity. In this study we addressed the effect of cholesterol enrichment on MPT induced by reactive oxygen species (ROS). Superoxide anion generated by xanthine plus xanthine oxidase triggered mitochondrial swelling and cytochrome *c* release in CHM, which was prevented by butylated hydroxytoluene, an anti-voltage-dependent anion channel antibody, or cyclosporin A. Furthermore, hydrogen peroxide generated by the combination of ganglioside GD3 and mitochondrial GSH depletion elicited mitochondrial swelling and release of cytochrome *c*, Smac/Diablo and apoptosis-inducing factor in control mitochondria and CHM. Thus, ROS induce MPT and apoptosome activation regardless of decreased mitochondrial membrane dynamics due to cholesterol enrichment.

Key words: Oxidative stress; Apoptosis; Caspase; Glutathione; Cholesterol; Mitochondrial glutathione; Ganglioside GD3

1. Introduction

The mitochondrial permeability transition (MPT), a process characterized by mitochondrial swelling, uncoupling and inner membrane permeabilization to small solutes, provides a mechanism for the mitochondrial regulation of cell death [1–3]. During this process several proteins that normally reside in the intermembrane and intracristal space are released into the cytosol; once there they can either assist in apoptosome assembly or disable inhibitor of apoptosis proteins, thus promoting cell death [4,5]. Apoptosis-inducing factor (AIF), another protein released during mitochondrial permeabilization, induces the characteristic morphological changes of apoptosis in a caspase-independent manner [6]. Although MPT constitutes a gateway for the release of these specialized mitochondrial proteins, they can also escape mitochondria in a MPT-independent fashion involving the selective permeabilization of the outer mitochondrial membrane without disruption of the inner membrane [7–10].

MPT is a multiprotein complex whose molecular composition and regulation is not entirely understood. A current model indicates the involvement and interaction of proteins at specific sites where the inner and outer mitochondrial membranes contact [11,12]. MPT can be regulated by several factors including Ca^{2+}, ADP levels, matrix pH, $\Delta\Psi_m$, mitochondrial energy status and lipid peroxidation [13–18]. In addition to these factors, reactive oxygen species (ROS) also regulate MPT by targeting specific thiol residues of certain MPT components, e.g. adenine nucleotide translocator (ANT) [19,20]. Furthermore, superoxide anion was shown to induce cytochrome *c* (cyt *c*) release by a voltage-dependent anion channel (VDAC)-dependent permeabilization of the outer mitochondrial membrane [21].

Glycosphingolipids regulate many cellular functions such as cell adhesion and signal transduction [22,23]. Particularly, ganglioside GD3 (GD3), a sialic acid-containing glycosphingolipid, has been identified as a lipid death effector [24]. GD3 interacts with mitochondria in intact cells in response to apoptotic stimuli and elicits a burst of ROS from the mitochondrial respiratory chain contributing to the release of proapoptotic proteins and apoptosome activation [25–28].

Recent studies have shown that cholesterol enrichment of isolated rat liver mitochondria (CHM) impaired the ANT-mediated MPT and subsequent release of mitochondrial proapoptotic proteins due to decreased membrane fluidity [29]. Thus, in the present study, we addressed the effect of cholesterol enrichment on ROS-induced MPT, monitored as mitochondrial swelling and subsequent release of proapoptotic proteins and apoptosome activation in a cell-free system.

2. Materials and methods

2.1. Reagents and antibodies

Atractyloside (ATR), cyclosporin A (CSA), cholesterol, *o*-phthalaldehyde, phenylmethylsulfonyl fluoride, CHAPS, pepstatin A, leupeptin, aprotinin, *N*-acetyl-leucyl-leucyl-norleucine, xanthine, xanthine

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Abbreviations: ANT, adenine nucleotide translocator; AIF, apoptosis-inducing factor; ATR, atractyloside; BHT, butylated hydroxytoluene; CHM, cholesterol-enriched mitochondria; CSA, cyclosporin A; cyt *c*, cytochrome *c*; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; GD3, ganglioside GD3; HP, (*R*,*S*)-3-hydroxy-4-pentenoate; mGSH, mitochondrial reduced glutathione; MPT, mitochondrial permeability transition; X+XO, xanthine plus xanthine oxidase; VDAC, voltage-dependent anion channel

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oxidase and butylated hydroxytoluene (BHT) were purchased from Sigma (St. Louis, MO, USA). 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) was from Molecular Probes (Eugene, OR, USA). GD3 was from Matreya (Pleasant Gap, PA, USA, 99% purity by thin layer chromatography). Carboxy-2′,7′-dichlorodihydrofluorescein diacetate methyl ester (carboxy-H_2DCFDA), dihydroethidium and anti-cytochrome oxidase subunit II were obtained from Molecular Probes. Anti-Smac/Diablo antibody was purchased from Calbiochem and anti-cyt *c* antibody (clone 7H8.2C12) from PharMingen (San Diego, CA, USA). Rabbit antiserum, anti-AIF and anti-VDAC antibody, Ab#25, were generous gifts from G. Kroemer (Centre National de la Recherche Scientifique, Villejuif, France) and Dr. Y. Tsujimoto (Osaka University Graduate School of Medicine, Osaka, Japan), respectively.

2.2. Mitochondrial preparation and cholesterol loading

Rat liver mitochondria were isolated from liver homogenates by differential centrifugation or rapid centrifugation through a Percoll density gradient as described in detail previously [30]. Enrichment and recovery of mitochondria were ascertained by the specific activity of succinic dehydrogenase and functional integrity assessed as the ADP-stimulated oxygen consumption using a Clark oxygen electrode with succinate as substrate. Mitochondrial cholesterol loading using a cholesterol–albumin complex and cholesterol determination were performed as detailed previously [29].

2.3. Depletion of mitochondrial GSH

(*R*,*S*)-3-Hydroxy-4-pentenoate (HP) was synthesized as described previously [31] and used to deplete mitochondrial reduced glutathione (mGSH) levels [32]. GSH was measured from mitochondria after trichloroacetic acid precipitation by high performance liquid chromatography as described [32].

2.4. ROS determination

Mitochondrial suspensions were exposed to xanthine plus xanthine oxidase (X+XO) or GD3 with or without preincubation with HP in the presence of CM-H_2DCFDA (1 μM, 505 nm excitation and 529 mm emission) or dihydroethidium (5 μM, 480 nm excitation and 620 nm emission) and fluorescence was measured in a fluorimeter as described previously [32].

2.5. Measurement of MPT

Large-amplitude swelling was measured spectrophotometrically by recording absorbance at 540 nm (A_{540nm}). Isolated rat liver mitochondria (1 mg/ml) were suspended in a buffer consisting of 200 mM sucrose, 10 mM Tris-MOPS, 5 mM succinate, 1 mM potassium phosphate, 2 μM rotenone, 1 μg/ml oligomycin, 10 μM EGTA, pH 7.4 at 25°C as described before [29]. A_{540nm} was registered in control mitochondria or CHM after addition of ATR (100 μM), X+XO (0.1 mM and 20 U/ml, respectively) or GD3 (10 μM) with or without CSA (5 μM).

2.6. Western blot analysis of cyt c, Smac/Diablo and AIF

Mitochondrial supernatants and pellets were collected by centrifugation at 10 000 × *g* for 5 min at 4°C. 20–25 μg proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (15% gel) and transferred to nitrocellulose filters. Blots were probed with anti-cyt *c* (mouse monoclonal antibody; clone 7H8.2C12, dilution 1:2000), anti-AIF (rabbit antiserum, dilution 1:2000) and anti-Smac/Diablo (rabbit polyclonal antibody, dilution 1:1000) and visualized with horseradish peroxidase-coupled secondary antibodies and ECL developing kit (Amersham Pharmacia Biotech, Rainham, UK). Parallel aliquots were analyzed by immunoblotting for the release of cytochrome oxidase using monoclonal antibody anti-cytochrome oxidase subunit II to confirm the specificity of mitochondrial protein release. In some cases mitochondria were treated with CSA (5 μM), BHT (50 μM), Ab#25 or control IgG antibody (0.5 μg/ml) and DIDS (100 μM) to assess their effect on ROS generation, MPT or the release of mitochondrial proteins induced by X+XO or GD3 (with or without mGSH depletion by HP).

2.7. Statistical analyses

Statistical analyses for comparison of mean values for multiple comparisons between mitochondrial preparations were made by one-way analysis of variance followed by Fisher's test.

3. Results and discussion

3.1. Superoxide anion induces mitochondrial swelling and release of cyt c independently of cholesterol content

MPT can be regulated by a number of factors including reactive oxygen and nitrogen species [19,20]. Recent observations in rat liver mitochondria have shown that cholesterol deposition in mitochondrial membranes impaired the ability of ATR to induce MPT and the release of apoptogenic proteins [29]. Thus, we tested the effect of cholesterol on the regulation of MPT and release of intermembrane proteins caused by ROS overgeneration. Exposure of rat liver mitochondria to a cholesterol–albumin complex resulted in a significant cholesterol deposition (4.3 ± 0.5 vs. 10.4 ± 1.2 μg cholesterol/mg protein in control mitochondria and CHM, respectively) compared to mitochondria incubated with albumin or cholesterol separately as described previously [29]. Isolated mitochondria with or without cholesterol loading were exposed to a superoxide anion-generating system, X+XO, examining changes in A_{540nm} and release of cyt *c*. The generation of ROS by X+XO was bimodal and similar in control mitochondria or CHM, with a three- to four-fold higher level of

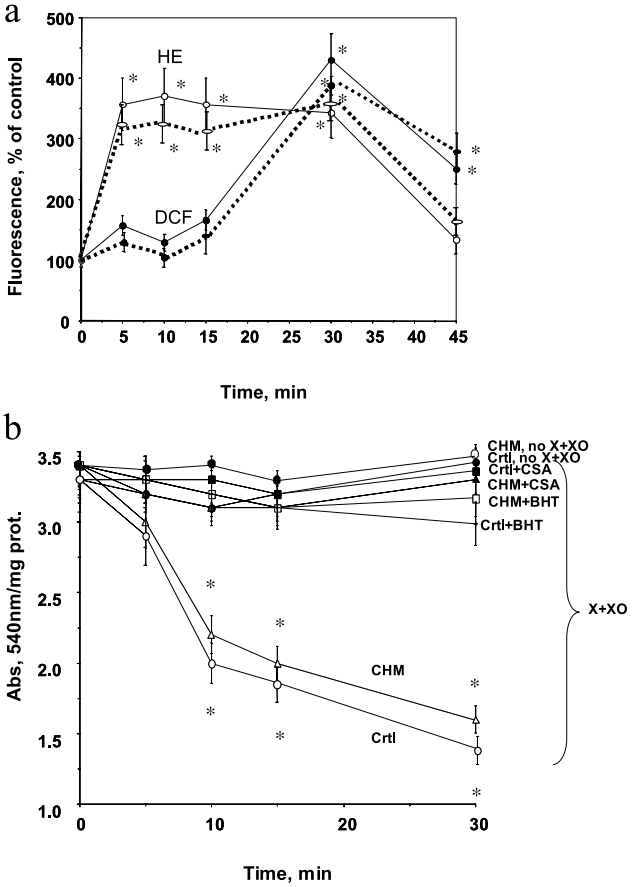

Fig. 1. Effect of $O_2^{\bullet-}$ on mitochondrial swelling. Control mitochondria and CHM (1 mg/ml) were incubated with X+XO to examine the time-dependent increase of dihydroethidium and DCF fluorescence (a). Changes in absorbance at 540 nm over time in response to X+XO (b) were determined in control mitochondria or CHM with or without incubation with CSA or BHT as indicated in Section 2. Solid and broken lines in panel a represent the changes in control mitochondria and CHM, respectively. Data are the mean ± S.D. of four individual experiments. *$P < 0.05$ vs. control without X+XO.

superoxide anion (determined as dihydroethidium fluorescence) with respect to control generated within the first 5–15 min of X+XO addition. However, while the level of superoxide anion decreased by 45 min, the levels of peroxides (determined as DCF fluorescence) increased above control by 30 min of incubation (Fig. 1a). Next, we determined the impact of ROS generated by X+XO on MPT. As seen the magnitude of swelling determined by loss of A_{540nm} caused by X+XO was similar in both control mitochondria and CHM (Fig. 1b). The effect of X+XO was accompanied by a comparable release of cyt *c* in both control mitochondria and CHM (Fig. 2a,b). This process was specific for cyt *c* as ascertained from the absence of cytochrome oxidase in the supernatant of mitochondrial suspensions (Fig. 2). As expected, cholesterol enrichment decreased mitochondrial membrane fluidity monitored from the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene and trimethylammonium 1,6-diphenyl-1,3,5-hexatriene (not shown) in agreement with recent observations [29]. Furthermore, CSA blocked X+XO-induced mitochondrial swelling (Fig. 1b) and cyt *c* release (Fig. 2a,b) to an equal extent in control mitochondria and CHM (Figs. 1b and 2).

Prior studies indicated that VDAC played a key role in the permeabilization of the mitochondrial outer membrane and subsequent release of cyt *c* induced by superoxide anion

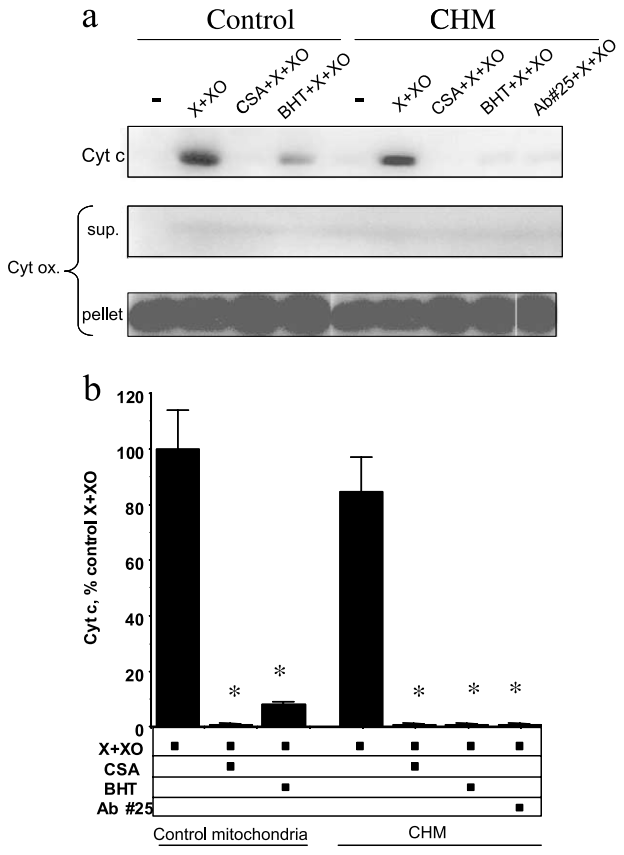

Fig. 2. Effect of $O_2^{\bullet-}$ on the release of cyt *c*. Cyt *c* and cytochrome oxidase (cyt ox.) were analyzed by Western blotting as described in Section 2 in supernatants and mitochondria (a). In some cases, mitochondria were preincubated with the antioxidant BHT (50 μM), the VDAC inhibitor Ab#25 (0.5 μg/μl) or CSA (5 μM) before the exposure to X+XO. The densitometric quantitation of cyt *c* release under these conditions (b) is the mean ± S.D. of four individual experiments. $^*P < 0.05$ versus control mitochondria or CHM treated with X+XO.

[21]. Thus, we tested whether VDAC was involved in our current observations with X+XO. Pretreatment of mitochondria with an antibody that interferes with the function of VDAC, Ab#25, characterized previously [33], blocked the cyt *c* release induced by X+XO (Fig. 1b); a control IgG antibody, however, had no effect (not shown). Similar results on the release of cyt *c* by X+XO were observed when mitochondria were treated with the VDAC blocker DIDS, in agreement with previous findings [21,34]. DIDS, however, did not interfere with the generation of ROS by X+XO, further documenting that its effect was mediated by targeting VDAC (not shown). Moreover, the inhibitory effect of the antioxidant BHT in X+XO-induced cyt *c* release (Fig. 2) and mitochondrial swelling (Fig. 1b) confirmed that the effect of X+XO was mediated by ROS production.

Thus, these data provide evidence that X+XO induces both mitochondrial swelling and cyt *c* release regardless of the cholesterol deposition and hence of decreased membrane fluidity and contrast with recent findings showing the dependence of ANT on appropriate mitochondrial membrane fluidity [29]. Some aspects, however, from our findings are in agreement with those of Madesh and Hajnoczky [21], namely, the involvement of VDAC in the permeabilization of mitochondria by X+XO as ascertained from the inhibitory effect of the specific anti-VDAC antibody used (Ab#25) both in isolated rat liver mitochondria (our present case) and in permeabilized HepG2 cells [21]. Moreover, DIDS, an anion inhibitor, mimicked the effect of the VDAC antibody #25. Prior studies have shown that DIDS as well as dicyclohexylcarbodiimide, a carboxyl-modifying agent known to bind specifically to mitochondrial VDAC [35,36], abolished the channel activity of VDAC reconstituted in planar lipid bilayers [34,37]. However, our findings as opposed to those of Madesh and Hajnoczky show that CSA prevents X+XO-induced A_{540nm} loss and release of cyt *c*. Differences in mitochondria from rat liver vs. HepG2 cells or in the fate and/or disposition of superoxide anion formed by X+XO between isolated mitochondria, as in the present study, compared to permeabilized HepG2 cells, in which the presence of subcellular organelles may limit the impact of superoxide anion on mitochondria, may account for this discrepancy. In addition, although X+XO-induced superoxide anion may target VDAC in the outer membrane [21], however, since CSA, in cases where it prevents MPT, functions by targeting cyclophilin D, an inner mitochondrial membrane protein (reviewed in [11]), it may be conceivable that X+XO-induced MPT may involve the participation of VDAC- and CSA-sensitive inner membrane MPT components.

3.2. GSH depletion and GD3 cooperate to induce MPT regardless of cholesterol deposition

To further verify the sensitivity of CHM to MPT induced upon ROS generation, we used GD3, a glycosphingolipid described previously to cause a burst of ROS from mitochondria [26,38]. Moreover, since the fate of mitochondrial ROS, particularly the disposition of hydrogen peroxide, is determined by the level of mGSH [24,39], we examined the influence of cholesterol loading on MPT induced by GD3 with or without mGSH depletion. Control mitochondria or CHM were first preincubated with HP which is transformed into a Michael acceptor that is conjugated with mGSH [31,32]. HP depleted mGSH levels to 20–30% of control values; the depleting po-

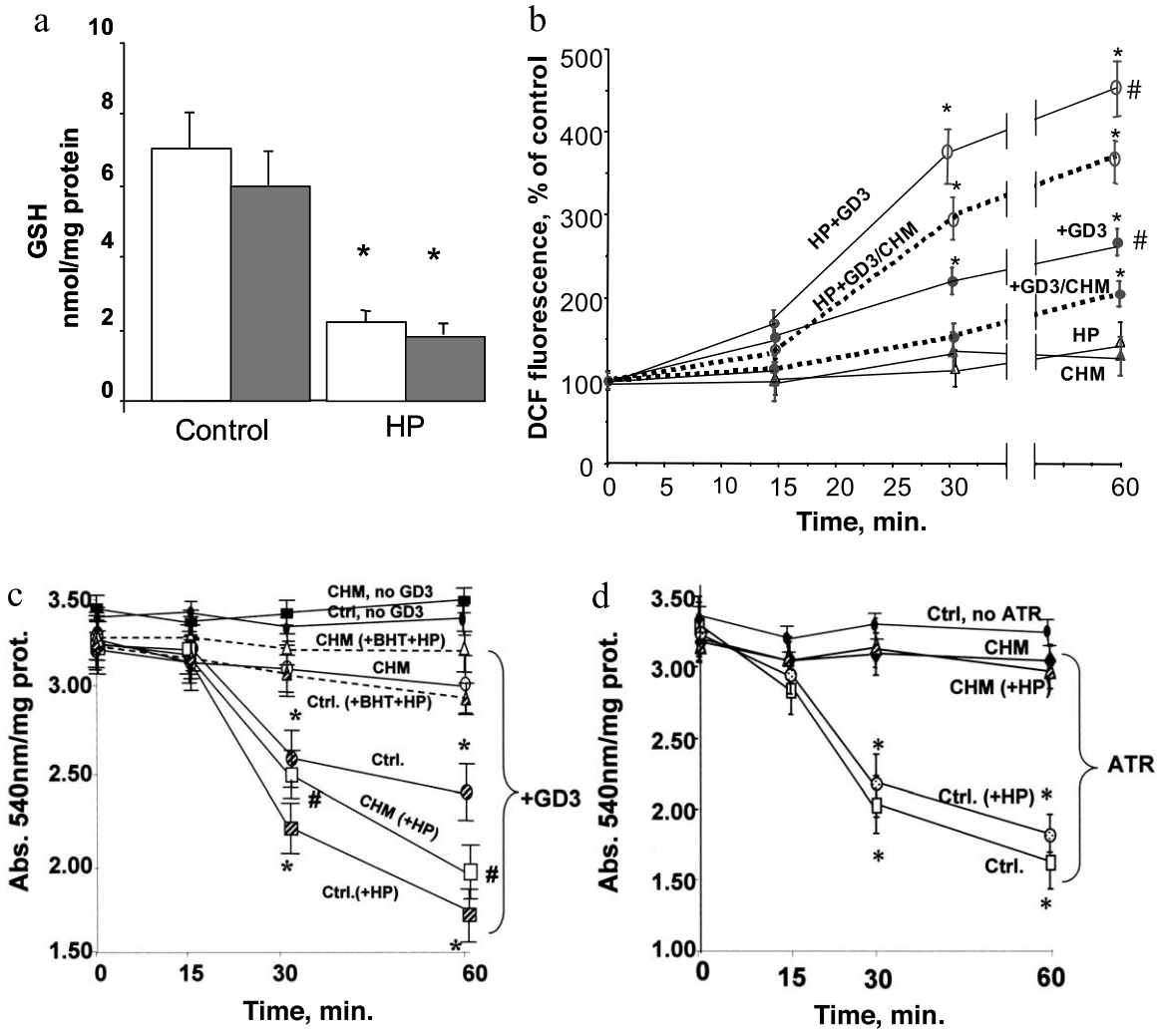

Fig. 3. Regulation by mGSH of MPT induced by ATR or GD3. Control mitochondria (open bars) or CHM (closed bars) (1 mg/ml) were incubated with 1 mM HP for 10 min at 37°C and after thorough washing, GSH levels were determined as described in Section 2 (a). The time-dependent generation of hydrogen peroxide was monitored from DCF fluorescence in the conditions shown (b). Control mitochondria or CHM were exposed to GD3 (10 μM) (c) or ATR (100 μM) (d) with or without HP preincubation to examine the changes in A_{540nm}. In some cases, mitochondria were pretreated with BHT before HP treatment and GD3 or ATR. Results are the mean ± S.D. of three individual experiments. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. corresponding CHM.

tential of HP was not affected by mitochondrial cholesterol content (Fig. 3a). While GD3 stimulated the formation of hydrogen peroxide in control mitochondria, monitored by the peroxide-sensitive fluorescence of DCF, this response was hampered by cholesterol loading (Fig. 3b). However, GSH depletion by HP further enhanced the generation of hydrogen peroxide by GD3 in control mitochondria or CHM compared to organelles not depleted of GSH although the fluorescence of DCF was lower in CHM than in control organelles (Fig. 3b). Moreover, to examine if the generation of hydrogen peroxide by GD3 under these conditions of cholesterol loading and GSH depletion was sufficient to induce MPT, we next monitored mitochondrial swelling. As seen (Fig. 3c), CHM did not show significant changes in A_{540nm} when exposed to GD3 in contrast to the outcome seen in control mitochondria (Fig. 3c). However, when GSH was depleted first by HP the loss of A_{540nm} elicited by GD3 was similar in both control mitochondria and CHM (Fig. 3c). The preincubation with the antioxidant BHT prevented mitochondrial swelling induced by GD3 in GSH-depleted mitochondria in both control and cholesterol-enriched organelles (Fig. 3c). Thus, the generation of hydrogen peroxide caused by GD3 correlates with MPT, suggesting a threshold above which hydrogen peroxide triggers MPT. Although GSH depletion is required for GD3 to achieve this response the impact of hydrogen peroxide on MPT is similar in control mitochondria and CHM indicating that the inability of CHM to undergo MPT in response to GD3 was due to the impaired generation of reactive species.

Since our prior observations indicated that the function of ANT as a MPT component is modulated by membrane fluidity [29], we examined the role of mGSH on ATR-mediated MPT (Fig. 3d). The depletion of mGSH did not modify the ATR-induced MPT as determined by the osmotic swelling of mitochondria nor the resistance seen in CHM (Fig. 3d). Furthermore, consistent with these findings, HP pretreatment did not affect the release of cyt *c*, Smac/Diablo or AIF induced by ATR (not shown). Finally, we examined the release of cyt *c*, Smac/Diablo and AIF by GD3 with or without mGSH depletion. As shown the data on osmotic swelling (Fig. 3c) paral-

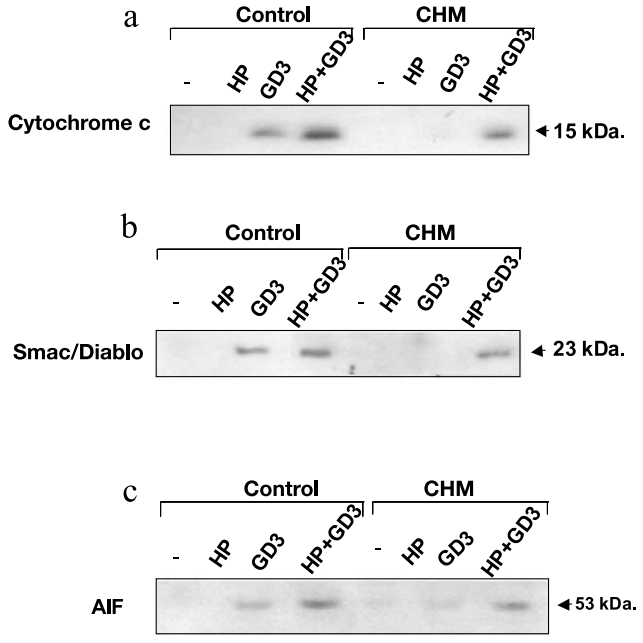

Fig. 4. Release of apoptogenic proteins by GD3 in control mitochondria and CHM. Supernatants from control mitochondria or CHM that were treated with GD3 with or without HP pretreatment were used to analyze the levels of cyt *c* (a), Smac/Diablo (b) or AIF (c) by Western blotting as described in Section 2. The blots shown are representative of four independent experiments showing similar results.

leled the release of apoptogenic factors caused by GD3 (Fig. 4). GD3 induced the release of cyt *c*, Smac/Diablo and AIF in control mitochondria that was enhanced upon mGSH depletion. However, in CHM, mGSH depletion was required for GD3 to induce the release of these proteins (Fig. 4). Consistent with the observations on mitochondrial swelling, BHT prevented the release of these proteins in mGSH-depleted mitochondria (not shown). In addition, the released proapoptotic factors from mitochondria under mGSH depletion and GD3 treatment resulted in the assembly of the apoptosome in a cell-free system determined from the fluorescence of caspase-3 substrates (not shown). Taken together these data highlight the differential role of mGSH in regulating MPT depending on the stimuli used. ATR, a c-conformation ligand of ANT, triggers MPT upon its binding to ANT. The ability of ATR to induce MPT through ANT is regulated by mitochondrial membrane dynamics [29]. The present observations indicate that mGSH depletion per se does not alter the dependence of ANT on appropriate membrane fluidity as CHM are resistant to ATR-mediated MPT regardless of whether mGSH was depleted or not. Moreover, although mGSH levels were considerably depleted by HP to around 2 nmol/mg protein (Fig. 3a), the magnitude of depletion was insufficient to stimulate peroxide generation. In line with this, it was shown that only when mGSH levels were depleted below 1 nmol/mg there was enhanced generation of hydrogen peroxide induced by antimycin A [40]. In contrast to these results with ANT, the ability of GD3 to induce MPT is dependent on the mGSH status. The combination of GD3 and mGSH depletion is more effective than GD3 alone to induce mitochondrial swelling and release of apoptogenic proteins and, more importantly, CHM are rendered sensitive to GD3 upon mGSH depletion (Fig. 3c). Therefore, the combination of mGSH depletion and a ROS generating stimulus, e.g. GD3, promotes apoptosome assembly independently of the cholesterol content of mitochondria.

The comparison of the ROS generation and impact on MPT between X+XO and GD3 is of interest and provides insight into the apparent resistance of CHM to GD3-induced MPT. The pattern of ROS generation by X+XO or the combination of GD3 plus HP agrees with the time-dependent MPT changes observed in either case (Figs. 1 and 3). Furthermore, while X+XO generates ROS from outside mitochondria, GD3 acts on the electron transport chain within mitochondria favoring the formation of hydrogen peroxide from Mn-SOD-catalyzed dismutation of superoxide anion as characterized previously [26,38]. Consistent with this different topology of ROS generation cholesterol loading modulates the generation of ROS by GD3 but not that caused by X+XO (Figs. 1a and 3b). The level of hydrogen peroxide elicited by GD3 in mitochondria with repleted GSH content reflects the stimulation of these species from the electron transport chain; although this rate is hampered by cholesterol through a mechanism that would deserve further investigation in the future, in conditions of GSH depletion hydrogen peroxide accumulates to a higher level due to impaired function of the GSH redox cycle of mitochondria [24,39]. Importantly, in this scenario of GSH depletion hydrogen peroxide elicits MPT in control mitochondria or CHM (Fig. 3c). Taken collectively, the generation of ROS either extramitochondrially or within mitochondria induces MPT independently of cholesterol loading.

3.3. Concluding remarks

The present study shows the differential effect of cholesterol deposition on the mitochondrial permeabilization, release of apoptogenic factors and subsequent apoptosome activation. Mitochondrial swelling and the inhibitory effect of CSA are characteristic features of MPT [2,11,12]. While cholesterol enrichment impairs ANT-mediated MPT and release of mitochondrial proteins, CHM undergo swelling and release apoptogenic proteins in response to stimuli that overproduce ROS as illustrated with X+XO or the combination of mGSH depletion and GD3 exposure. These findings may be of relevance in the regulation of apoptotic pathways in conditions of stimulated cholesterol deposition in mitochondria. For instance, mitochondria from alcohol-fed rat liver or acetaldehyde-treated HepG2 cells have higher cholesterol levels and defective transport of GSH into mitochondria [41–43]. In this scenario alcohol intake would stimulate ganglioside biosynthesis from sphingosine recycling, described recently in cerebellar granule cells cultured with ethanol [44], which in combination with selective mGSH depletion would lead to mitochondrial cell death, providing the basis for the sensitivity of hepatocytes from alcohol-fed rats to tumor necrosis factor-induced cell death [45,46].

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