Commitment to Apoptosis by GD3 Ganglioside Depends on Opening of the Mitochondrial Permeability Transition Pore*

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We have studied the effects of GD3 ganglioside on mitochondrial function in isolated mitochondria and intact cells. In isolated mitochondria, GD3 ganglioside induces complex changes of respiration that depend on the substrate being oxidized. However, these effects are secondary to opening of the cyclosporin A-sensitive permeability transition pore and to the ensuing swelling and cytochrome c depletion rather than to an interaction with the respiratory chain complexes. By using a novel in situ assay based on the fluorescence changes of mitochondrially entrapped calcein (Petronilli, V., Miotto, G., Canton, M., Colonna, R., Bernardi, P., and Di Lisa, F. (1999) Biophys. J. 76, 725–734), we unequivocally show that GD3 ganglioside also induces the mitochondrial permeability transition in intact cells and that this event precedes apoptosis. The mitochondrial effects of GD3 ganglioside are selective, in that they cannot be mimicked by either GD1a or GM3 gangliosides, and they are fully sensitive to cyclosporin A, which inhibits both the mitochondrial permeability transition in situ and the onset of apoptosis induced by GD3 ganglioside. These results provide compelling evidence that opening of the permeability transition pore is causally related to apoptosis.

Mitochondria are under close scrutiny as regulators of apoptosis and as potential targets for therapeutic intervention in the context of both accidental and programmed cell death (for reviews, see Refs. 1 and 2). One apoptotic pathway where mitochondria may play a role is that involving sphingomyelin metabolism. This pathway is activated by a wide variety of stimuli and gives rise to messenger molecules that include ceramides and gangliosides (for reviews, see Refs. 3 and 4). Ceramides have been reported to affect various aspects of mitochondrial function in isolated mitochondria and intact cells (5–8); and GD3 ganglioside, an essential proapoptotic component of the ceramide pathway, has been shown to depolarize mitochondria in situ independent of caspase inhibition (9).

One target for mitochondrial apoptotic signaling is the MTP, an inner membrane channel that has been the subject of intensive investigation in bioenergetics (for a recent review, see Ref. 10). The idea that the MTP may be involved in cell death was introduced in the early 1990’s (11–13), and it has gained momentum with recent work suggesting that MTP-dependent swelling may be causally linked to release of intermembrane apoptosis-inducing factor (14) and possibly of cytochrome c, a point that is still controversial (15–17).

In this study we have characterized the interactions of GD3 ganglioside with isolated mitochondria and intact cells. We show that GD3 ganglioside has complex effects on respiration that can be entirely explained by onset of the PT and by the ensuing depolarization, depletion of pyridine nucleotides, swelling and loss of cytochrome c. By using a novel protocol for MTP detection in intact cells that is based on the fluorescence changes of mitochondrially entrapped calcein (18), we show that (i) GD3 is a selective inducer of the PT in situ and (ii) both MTP opening and the ensuing apoptosis are inhibited by CsA. These results provide direct evidence that mitochondrial depolarization by GD3 ganglioside is due to a PT and that this event is causally related to apoptosis.

MATERIALS AND METHODS

Rat liver mitochondria from albino Wistar rats weighing about 300 g were prepared by standard centrifugation techniques (19). Mitochondrial absorbance changes were followed at 540 nm with a Perkin-Elmer Lambda-10 spectrophotometer, and oxygen consumption was determined polarographically using a Clark-type oxygen electrode. All assays were performed at 25 °C in instruments equipped with thermostatic control and magnetic stirring.

MH1C1 rat hepatoma cells were seeded onto uncoated 22-mm diameter round glass coverslips and grown for 2 days in Ham’s F-10 nutrient mixture supplemented with 20% fetal calf serum in a humidified atmosphere of 95% air, 5% CO2 at 37 °C in a Forma tissue culture water-jacketed incubator.

For calcein staining, MH1C1 cells grown on coverslips were loaded for 20 min at 37 °C with 1 μM calcein-acetomethoxyl ester dissolved in 0.5 ml of Dulbecco’s modified phosphate-buffered saline without penicillin (Sigma D6650) supplemented with 1 mM CoCl2 (18). After loading, cells were washed free of calcein and Co2+ using a 1:25 (v/v) mixture supplemented with 20% fetal calf serum in a humidified atmosphere of 95% air, 5% CO2 at 37 °C in a Forma tissue culture water-jacketed incubator. For calcein staining, MH1C1 cells grown on coverslips were loaded for 20 min at 37 °C with 1 μM calcein-acetomethoxyl ester dissolved in 0.5 ml of Dulbecco’s modified phosphate-buffered saline without penicillin (Sigma D6650) supplemented with 1 mM CoCl2 (18). After loading, cells were washed free of calcein and Co2+ and maintained in the modified phosphate-buffered saline. Because the extent of mitochondrial loading with probes can be affected by the activity of the multidrug resistance P-glycoprotein (20), CsA was added to the cells after probe loading, and calcein fluorescence acquisition was started 20 min later. For staining with annexin-V, each coverslip was incubated for 15 min at 25 °C in 0.5 ml of a solution containing 140 mM NaCl, 5 mM CaCl2 and 10 mM Hepes/NaOH, pH 7.4, 2 μM propidium iodide, and annexin-V-FLUOS (Roche Molecular Biochemicals) to a final dilution of 1:25 (v/v). Cells were washed twice with phosphate-buffered saline before analysis.

Cellular fluorescence images were acquired with an Olympus IMT-2 inverted microscope, which was equipped for epifluorescent illumination and included a xenon light source (75 W) and a 12-bit digital-cooled CCD camera (Micromax, Princeton Instruments). For detection of calcein fluorescence, a filter set was used that is specifically designed to minimize bleed-through from propidium iodide and annexin-V fluorescence. For detection of propidium iodide and annexin-V, a filter set was used that is specifically designed to maximize the green fluorescence region. For calcein and propidium iodide, the filters used were a 510 ± 20-nm bandpass filter and a 695 ± 30-nm longpass filter, respectively. For annexin-V, the filters used were a 520 ± 10-nm bandpass filter and a 630 ± 30-nm longpass filter. All filters were from Chroma Technology. The specific filter sets were selected to cover the fluorescence spectrum of the dyes (for a recent review, see Ref. 7). The filter sets were used to acquire images of the cells that were then superimposed in each case after proper pixel normalization. For each condition, at least 100 cells were scored.}

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1 The abbreviations used are: MTP, mitochondrial permeability transition pore; PT, permeability transition; CsA, cyclosporin A; FCCP, carbonyl cyanide-p-trifluoromethoxyphenyl hydrazone; MOPS, 4-morpholinepropanesulfonic acid.
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**RESULTS**

The experiments of Fig. 1A document the effects of GD3 ganglioside on the rate of respiration maintained by rat liver mitochondria oxidizing glutamate plus malate (trace a) or succinate in the presence of rotenone (trace b). In both cases the addition of 7.5 μM GD3 ganglioside caused a modest increase in the rate of respiration, which was more marked with succinate as the substrate. Upon addition of the protonophore FCCP, a major difference emerged depending on the substrate being oxidized. With glutamate plus malate no further respiratory stimulation occurred unless both NADH and cytochrome c were added (trace a), whereas neither NADH nor cytochrome c alone had a major effect (not shown). With succinate, on the other hand, the expected stimulation of respiration was observed, but the effect of FCCP was transient and could only be restored by the addition of cytochrome c (trace b) irrespective of whether NADH had also been added (results not shown). All of these findings can be easily explained by induction of a PT, which causes immediate depolarization, rapid release of pyridine nucleotides, and swelling (see Fig. 2) with gradual depletion of cytochrome c. Indeed, in the presence of CsA the basal rate of respiration was unaffected by GD3 ganglioside irrespective of the substrate being oxidized, and the addition of FCCP caused the expected increase of respiration (Fig. 1B), which could be stimulated only marginally by the addition of NADH and cytochrome c (results not shown; see Ref. 21).

In the experiments of Fig. 2, we addressed the issue of specificity in protocols where onset of the PT was assessed based on a swelling assay. After the addition of a small amount of Ca^{2+} that was not sufficient to induce the PT per se, EGTA was added to prevent Ca^{2+} efflux on the uniporter (22) followed by 7.5 μM gangliosides (panel A). It can be seen that swelling followed the addition of GD3 (trace a) but not of GD1a (trace b) or of GM3 ganglioside (trace c) and that it was completely prevented by CsA (trace d). Panel B reports the concentration dependence for the effects of GD3 ganglioside, which could be reproducibly observed in the submicromolar range. It is worth mentioning that MTP opening was also prevented by the newly discovered inhibitors ubiquinone 0 and decylubiquinone (23) (results not shown).

In the experiments of Fig. 3 we tested whether MTP opening by GD3 ganglioside could also be observed in situ with the recently developed calcine-trapping/Co^{2+}-quenching technique. In these protocols calcine can be visualized inside mitochondria in intact cells, and a CsA-sensitive decrease of mitochondrial calcine fluorescence allows visualization of MTP openings even when these cannot be detected by potentiometric fluorescent probes (18). It can be clearly appreciated that GD3 ganglioside caused a marked decrease of mitochondrial calcine fluorescence along with its intracellular redistribution (A, A’, and closed circles in panel D), that these fluorescence changes were prevented by pretreatment with CsA (B, B’, and open circles in panel D), and that GD1a ganglioside did not cause measurable fluorescence changes (C, C’, and closed squares in panel D).

We finally asked the question of whether GD3 ganglioside was also able to induce cell death in our system. In the experiments of Fig. 4 we monitored the appearance of annexin V-

**Fig. 1.** Effects of GD3 ganglioside on mitochondrial respiration. The incubation medium contained 150 mM KCl, 10 mM Tris-MOPS, 1 mM P_{1}, 10 μM EGTA-Tris, and 5 mM glutamate-Tris plus 2.5 mM malate-Tris (traces a) or 5 mM succinate-Tris plus 2 μM rotenone (traces b). In the experiments of panel B, the incubation media were supplemented with 2 μM CsA. Final volume 2 ml, pH 7.4, 25 °C. The experiments were started by the addition of 1 mg of mitochondria (RLM). Where indicated (arrows), 7.5 μM GD3 ganglioside, 200 nM FCCP, 10 μM cytochrome c (cyt c) and 10 μM NADH were added.
reactive phosphatidylserine on the membrane surface, one of the earliest events in commitment to apoptosis (24). The experiment documents that treatment with GD3 ganglioside greatly increased the number of annexin-V positive (\(\text{panel B}\)) cells (compare with \(\text{panel A}\)), which was instead unaffected by treatment with GD1a ganglioside (\(\text{panel C}\)), and that CsA prevented the increase of annexin-V-positive cells, which was otherwise caused by GD3 ganglioside (\(\text{panel D}\)). These findings are quantitatively analyzed in \(\text{panel E}\) (closed columns), which also reports the number of cells that were positive for both annexin-V and propidium iodide staining (open columns).

**DISCUSSION**

The results of this paper provide compelling evidence that opening of the MTP is causally related to GD3-dependent apoptosis. We have shown that GD3 ganglioside is an inducer of the mitochondrial PT both in isolated rat liver mitochondria and in MH1C1 cells, a stabilized rat hepatoma line. The effects...
of GD3 ganglioside are selective in that they were not observed with the structurally related gangliosides GM3 and GD1a, and they can be assigned to a direct effect on the MTP. Indeed, and despite the apparent major effects of GD3 ganglioside on mitochondrial respiration and coupling, all of our results can be explained by pore opening rather than by inhibitory effects on the respiratory complexes or by unspecific changes of membrane permeability. Indeed (i) all of the effects of GD3 ganglioside on respiration could be prevented by CsA (Fig. 1); and (ii) despite their reported membrane-perturbing activity (23), gangliosides GM1 and GD1a lacked any permeabilizing effects both in intact mitochondria and in MH1C1 cells (this study).

Our results nicely complement a recent set of data on the involvement of GD3 ganglioside in ceramide-dependent apoptosis (26–28). It becomes now clear that the mitochondrial depolarization in situ previously reported by Testi and coworkers (9) can be easily explained by induction of MTP opening. In parallel measurements with tetramethylrhodamine methyl ester as the probe (29), we have confirmed that addition of GD3 ganglioside caused the depolarization, which is expected when the PT takes place (results not shown). Yet, it must be stressed that MTP openings of short duration may not be resolved by potentiometric probes, as we have documented in a recent study (18).

Although MTP opening was favored by the accumulation of low concentrations of Ca\(^{2+}\) (comparative results not shown, but see Fig. 2), it is noteworthy that the inducing effects of GD3 did not require added Ca\(^{2+}\). This property is only shared by the vicinal dithiol reagent, phenylarsine oxide (30, 31), and by the peptide mastoparan (32), which are among the most potent pore inducers. It is tempting to speculate that the levels of endogenous GD3 ganglioside, or its synthesis by the Golgi apparatus (33, 34), may contribute to determine the involvement of mitochondria in different models of cell death or in different cell types. This hypothesis is under active investigation in our laboratories, together with the definition of the site of interaction of GD3 ganglioside with the MTP and the structural requirements for its inducing effects.

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