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Mitochondria and cells produce reactive oxygen species in virtual anaerobiosis: relevance to ceramide-induced apoptosis

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Abstract Observations of apoptosis in virtual anaerobiosis have raised doubts on the significance of reactive oxygen species in the cascade of events of programmed cell death. This work presents evidence that cells and mitochondrial preparations produce similar levels of hydrogen peroxide under either aerobic or virtually anaerobic conditions. These levels are relevant to the increased production of radicals induced by a ceramide analog that promotes apoptosis. This ceramide acts at center o of mitochondrial complex III.

Key words: Reactive oxygen species; Mitochondrion; Apoptosis; Ceramide

1. Introduction

Programmed cell death or apoptosis follows multiple pathways that often vary in different cells [1–4]. Despite this diversity, most pathways of apoptosis seem to converge towards a central execution program in which mitochondria play a critical role [2,3,5]. In particular, alterations in mitochondrial function are seen in the early stages of apoptotic pathways involving ceramide [6–12]. The major mitochondrial alterations in apoptosis are a decrease in membrane potential and an increased production of reactive oxygen species (ROS) [2,3,6–10]. The relationship between these events is unclear, and presently controversial [1–5,8–10]. A current view is that the mitochondrial production of ROS could be an epiphenomenon in the apoptosis cascade [4,13,14]. This view is sustained by the observations that some pathways of apoptosis occur with cells maintained under conditions of virtual anaerobiosis [13,14]. The assumption in the interpretation of these observations is that mitochondria should produce very few ROS when cells are exposed to very low ambient concentrations of oxygen [4,13,14].

The results presented here suggest that this assumption is flawed. Mitochondria and cells do produce significant levels of hydrogen peroxide, a most dangerous ROS [15,16], in virtual anaerobiosis. Hydrogen peroxide production increases after treatment with an apoptogenic ceramide analog that inhibits center o of mitochondrial complex III. Thus, new evidence is provided for the involvement of mitochondrial ROS in ceramide-induced apoptosis.

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Abbreviations: DCF, dichlorofluorescein; DCFDA, dichlorodihydrofluorescein diacetate; DPI, diphenyleneiodonium; MOA, methoxyacrylate; Q, ubiquinone; ROS, reactive oxygen species; SMP, submitochondrial particles

2. Materials and methods

2.1. Materials and determinations

Methoxy-acrylate-stilbene (MOA-stilbene) was provided by Prof. P. Rich, Glynn Laboratory of Bioenergetics, University College of London, UK. Optical and fluorescent probes were purchased from Molecular Probes, Eugene, USA and determined in methanol (www.probes.com). Diphenyleneiodonium chloride (DPI) was purchased from Tocris Cookson, Bristol, UK. All other reagents were from Sigma Co., St. Louis, USA. The concentration of the inhibitors was determined spectrophotometrically [17,18]. Submitochondrial particles (SMP) from beef heart were prepared with the procedure of Hansen and Smith [19]. COS-7 cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle medium supplemented with 10% (w/v) fetal calf serum and 2.5% horse serum and counted microcytometrically. Before the experiments, cells were cultured in 24-well Nunc plates for one or two days in the same medium, washed twice with PBS, supplemented with PBS and then used for the microplate assays.

2.2. Enzyme assays

The activities of NADH-ubiquinone (Q) reductase and cytochrome *c* reductase were assayed as described previously [17]. Oxygen consumption was measured with an oxygraph (Strathkelvin Instruments, Glasgow, UK) under the same conditions as those in the ROS assays. The electric membrane potential generated by SMP was measured with oxonol VI [18]. Reduction of cytochrome *b* was monitored at 563–575 nm in an Aminco spectrophotometer [20]. Cell viability was evaluated by following the release of lactate dehydrogenase [13] or the quenching in the fluorescence of oxidized dichlorodihydrofluorescein diacetate (DCFDA) consequent to cell death [21].

2.3. ROS assays

The production of ROS in submitochondrial particles and cells was measured with a modified version of the methods with the DCFDA probe [9,10,21–25] using plate readers. To a final volume of 0.15 ml (in 96-well plates) or 0.42 ml (in 24-well plates) each well contained phosphate or PBS buffer, 1 μM DCFDA and 0.2–0.5 mg/ml (biuret) of SMP or 50–100 000 COS-7 cells. Cells were assayed with either endogenous substrates or after addition of 20 mM glucose in PBS. In some experiments, succinate and 0.2 mg/ml of SMP were added together with the cells to produce virtual anaerobiosis – this did not induce significant changes in cell viability, at least within 1–2 h.

Fluorescence measurements were accumulated every min for 1 h at 30°C with either a Molecular Dynamics Biolumin 920 plate reader (excitation at 485 nm and emission at 520 nm, with 5 nm bandwidths) or a Fluoroscan Ascent plate reader (excitation at 485 nm and emission at 538 nm). The quantitative evaluation of the fluorescence readings was undertaken with serial dilution of determined solutions of dichlorofluorescein (DCF) [22–24]. Calibration with hydrogen peroxide was avoided in view of the very slow reaction between DCFDA and hydrogen peroxide in the absence of biological catalysts (cf. [23,24]). This slow reaction has not been considered previously, leading to large under-estimations of the DCFDA response to hydrogen peroxide [9,26]. In some long-term experiments, DCFDA oxidation was followed spectrophotometrically at 504 nm to avoid the saturation in the fluorescence measurements above 400 nM.

3. Results

The increase in fluorescence and absorbance of the DCFDA probe mainly derives from reaction with hydrogen peroxide,

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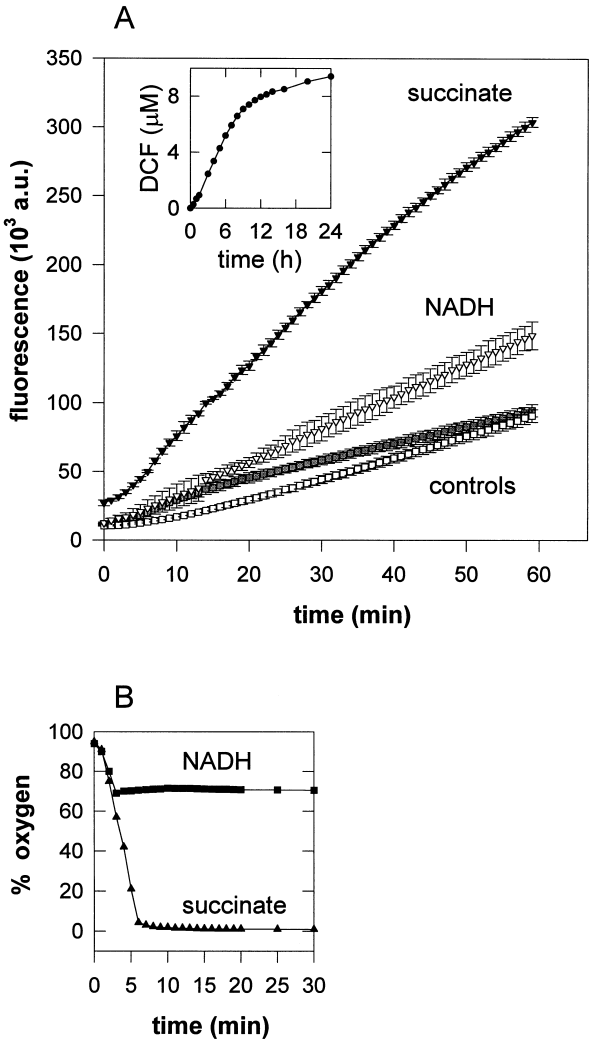

Fig. 1. Production of hydrogen peroxide in submitochondrial particles. Beef submitochondrial particles (0.5 mg/ml) were frozen-thawed to limit coupling and preincubated with 10 mM succinate (filled symbols) or supplemented with 0.2 mM NADH just before the fluorescence readings (empty symbols). In the sample with succinate and antimycin, the inhibitor (1 nmol/mg) was added after 8 min incubation with succinate, when the oxygen concentration was ca. 2%. In A the time-course of DCFDA oxidation is shown. The measurements were carried out in triplicates with the Biolumin reader, where 1 nM DCF was equivalent to 4×10^3 arbitrary units (a.u.). The insert shows the extended time-course of DCF production in the presence of antimycin, which was obtained by following the absorbance at 504 nm with an excess of DCFDA (10 μM). The oxygen consumption of the control samples is shown in B.

since it is about 70% sensitive to catalase (results not shown, cf. [23,24]). Fig. 1 shows the production of hydrogen peroxide in SMP treated with antimycin, the inhibitor of complex III (ubiquinol-cytochrome *c* reductase) that is well known to stimulate ROS production in mitochondria [15,27–29]. The samples were preincubated with succinate for enough time to attain near anaerobiosis, also before the addition of antimycin. Very similar results were obtained without this preincubation, whereas NADH oxidation produced less hydrogen peroxide than succinate oxidation (Fig. 1). NADH respiration induced a 30% reduction of the oxygen in the medium, whereas succinate respiration consumed virtually all the oxygen within 10 min (Fig. 1B). At the times corresponding to the fluorescence measurements, the oxygen tension was below the limit of detection of the oxygen electrode (i.e. $<1\%$). The production of hydrogen peroxide in SMP lasted for over one day, until completion of DCFDA oxidation (Fig. 1A, insert).

The results shown in Fig. 2 indicated that the level of hydrogen peroxide measured with SMP was comparable to that produced by live cells under the same conditions. When SMP were added to live cells for eliminating oxygen via succinate respiration, the cumulative production of hydrogen peroxide was larger than the sum of that with either SMP or cells in separate wells. Presumably, cells under anoxic conditions produced effectors such as ceramide [30] that could diffuse to the SMP and enhance their production of radicals. Although SMP alone reduced most oxygen with succinate respiration (cf. Fig. 1), cells without SMP had a normal concentration of oxygen in the medium, since their respiration was restricted by the availability of endogenous substrates. Moreover, in the presence of respiratory inhibitors such as myxothiazol – which, contrary to antimycin, did not enhance ROS production (Fig. 3, cf. [29]) – the concentration of oxygen changed little in both cells and SMP. Nevertheless, the production of hydrogen peroxide by cells and SMP treated with myxothiazol or MOA-stilbene was similar to the control values (Fig. 2).

To address the question of whether the observed levels of hydrogen peroxide were relevant to the increased production of ROS in apoptotic cells [2–4,6–10,31], we have used the *N*-

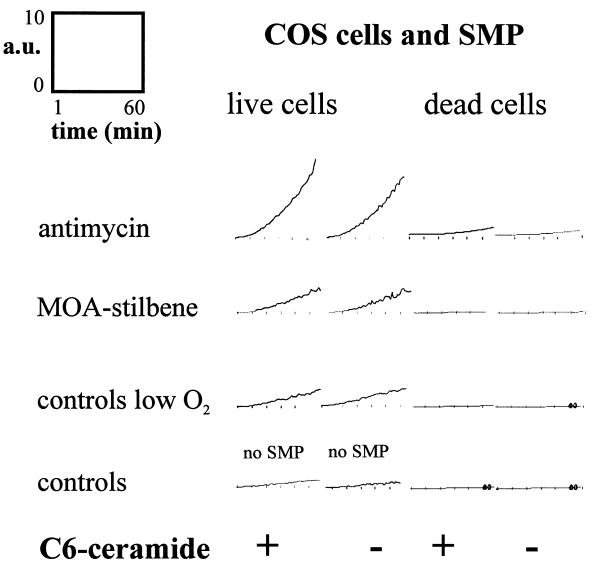

Fig. 2. Production of hydrogen peroxide of COS cells and SMP. COS cells (50 000 per well) in PBS were mixed together with 0.2 mg/ml SMP and assayed for hydrogen peroxide production after 10 min incubation with 10 mM succinate, followed by 10 min incubation with 12 μM C6-ceramide and inhibitors (or equivalent volumes of DMSO in the controls). The concentration of antimycin was 0.5 μM and that of MOA-stilbene 2 μM. The measurements were carried out with the Fluoroscan plate reader, where 1 a.u. of fluorescence was equivalent to 12.5 nM DCF. Virtual anaerobiosis of the samples with SMP was attained at about 8 min after succinate addition (cf. Fig. 1B), as verified also from the decay of the membrane potential in SMP alone. Dead COS-7 cells were obtained by multiple cycles of freezing and thawing in PBS. The rates of fluorescence increase with SMP alone were nearly superimposable to those obtained in the presence of dead cells. In fact, cell death essentially abolished oxidation of DCFDA (cf. [21]).

hexanoyl analog of ceramide (C6-ceramide). This cell-permeant ceramide induces rapid apoptosis [10,11] with ROS production at the mitochondrial level [9,10]. C6-ceramide did not induce ROS production in blanks or with dead COS cells (Fig. 2 and results now shown), but stimulated the hydrogen peroxide production by antimycin-treated SMP and live COS cells (Figs. 2 and 3). The increase in ROS production by the combination of C6-ceramide and antimycin (Fig. 2) was consistent with previous evidence [6,9,10]. On the other hand, C6-ceramide induced less hydrogen peroxide production in inside-out SMP than in intact mitochondria (results not shown), probably because mitochondria contained endogenous peroxidases facilitating the oxidation of DCFDA (cf. [21,24]).

With endogenous substrates, C6-ceramide did not substantially increase the basal cellular production of hydrogen peroxide (Fig. 2). However, with external nutrients such as glucose C6-ceramide increased ca. 3-fold the rates of hydrogen peroxide production by COS cells (Fig. 3). Fig. 3 also shows

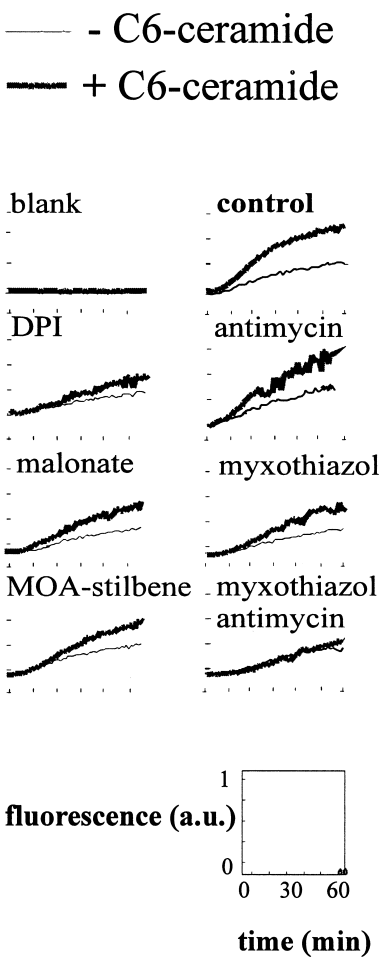

Fig. 3. Ceramide stimulation of hydrogen peroxide production in cells. The experiment was carried out in two parallel 24-well plates in the Fluoroscan instrument under conditions similar to those in Fig. 2, except that no SMP and succinate were present. After 10 min incubation with DCFDA, the cells were supplemented with 20 mM glucose just before the fluorescence measurements. The concentration of the inhibitors were: 12 μM for C6-ceramide, 0.5 μM for antimycin, 2 μM for MOA-stilbene, 1 μM for myxothiazol, 10 mM for malonate and 25 μM for DPI. The control rates were 0.085 nM/min without ceramide (thin traces) and 0.226 nM/min with ceramide (thick traces).

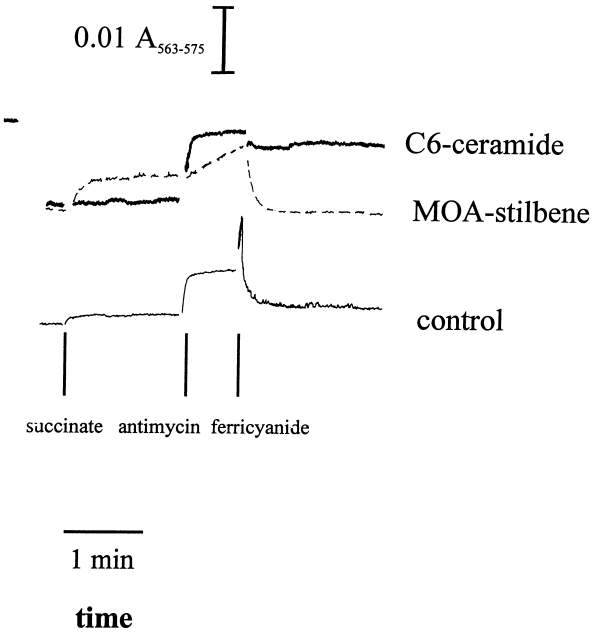

Fig. 4. Effect of C6-ceramide on the redox state of mitochondrial cytochrome *b*. Frozen-thawed SMP (1.2 mg/ml) were reduced with 5 mM succinate and supplemented with 1 nmol/mg of antimycin at the times indicated by the vertical bars. After the reduction of cytochrome *b* reached near equilibrium, 50 μM K-ferricyanide was added to elicit a transient extra-reduction of the cytochrome, which was followed by partial re-oxidation. C6-ceramide (solid trace) and MOA-stilbene (dashed trace) were added at 25 and 2 μM, respectively. The level of dithionite reduction cytochrome *b* in the ceramide-treated sample is shown in the left. At the same concentration, C6-ceramide decreased by over 70% the rate of NADH-cytochrome *c* reductase in frozen-thawed SMP.

the effect of various mitochondrial inhibitors on the rates of hydrogen peroxide production in glucose-supplemented cells. Dehydrogenase inhibitors such as DPI [32] and malonate decreased ROS production in untreated cells as in ceramide-treated cells (Fig. 3). Antimycin had apparently little effect over the controls, but other complex III inhibitors such as myxothiazol and MOA-stilbene substantially decreased the rate and extent of hydrogen peroxide production, especially in the presence of C6-ceramide (Fig. 3). Interestingly, the combination of myxothiazol and antimycin was particularly effective in quenching the production of ROS in cells treated with C6-ceramide (Fig. 3).

Complex III has two Q reaction sites: center i that is specifically inhibited by antimycin and center o that is inhibited by several compounds including myxothiazol and MOA-stilbene [33,34]. Recently, C6-ceramide has been shown to inhibit the activity of complex III at the concentrations that induce apoptosis [35]. The analysis of the effect of complex III inhibitors on the production of ROS (Fig. 3) suggested that center o could be the site of ceramide inhibition in complex III. We thus tested ceramides on the oxidant-induced extra-reduction of cytochrome *b*, a reaction which is characteristically inhibited by center o inhibitors in antimycin-treated complex III [33]. As shown in Fig. 4, C6-ceramide effectively eliminated the extra-reduction of cytochrome *b* by ferricyanide in antimycin-treated SMP. The ceramide analog also induced an inhibition of the subsequent re-oxidation of cytochrome *b* that was more pronounced than that observed with MOA-stilbene or myxothiazol (Fig. 4, cf. [20]).

4. Discussion

This work provides novel information indicating that mitochondria and cells produce substantial amounts of hydrogen peroxide under conditions of virtual anaerobiosis (Figs. 1 and 2). Two major reasons can explain how mitochondrial enzymes sustain a continuous production of hydrogen peroxide even after most of the oxygen has been consumed. The first reason is that submicromolar concentrations of oxygen that remain in the medium after succinate respiration still react with reduced low-potential groups, e.g. iron-sulfur clusters or semiquinones, thereby producing nanomolar quantities of superoxide and hydrogen peroxide [31]. The second reason is that the abundant reduced iron in mitochondria catalyzes self-sustaining Fenton reactions with the hydrogen peroxide formed by substrate oxidation [15,16,25].

Previously, the virtual removal of oxygen from the ambient culture conditions of cells has been assumed to annihilate the production of ROS by mitochondria, mostly in view of the reduced levels of lipid and protein oxidation [13,14]. The assays of lipid and protein peroxidation used in [13,14] are sensitive to micromolar levels of oxidized material that are produced only at normal concentrations of oxygen [16,31,36]. On the other hand, the physiological levels of ROS such as hydrogen peroxide are in the nanomolar range, as shown here (Figs. 2 and 3). Because sensitive assays of hydrogen peroxide have not been carried out in previous studies [13,14], it is likely that the cellular levels of this crucial ROS have escaped detection. Hence, the conclusion that mitochondrial ROS are not necessary in apoptosis because cells in virtual anaerobiosis follow normal death programs [4,13,14] is based on a flawed assumption. Cells do produce hydrogen peroxide in anaerobiosis like in aerobiosis, and mitochondria are the major source of these radicals (Figs. 2 and 3, cf. [3,6,9,10]).

Ceramide is a second lipid messenger that is apparently involved in many pathways of cellular stress and apoptosis and thus has multiple actions in cells (see [37] for a review). Recently, mitochondrial complex III has been identified as a ceramide target since its activity is inhibited by analogs such as C6-ceramide [35]. The results presented here clarify that center o is the site of action of C6-ceramide in mitochondrial complex III. Of note, center o function is modulated by the redox and conformational state of the Rieske iron-sulfur protein [38], which is able to react with oxygen and produce ROS [39]. Presumably, the interaction of ceramides perturbs the conformation of the domain of complex III that protrudes at the cytoplasmic side of the inner mitochondrial membrane, where the Rieske protein and the docking site of cytochrome *c* are located [38]. Given the critical role of cytochrome *c* release in the execution of apoptosis [3,5,40], there may be some potential links between the action of ceramide at center o and complex III binding of cytochrome *c*. The solution of the complete crystal structure of complex III [38] will hopefully provide insights to understand the molecular aspects of ceramide interaction.

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