Paclitaxel Induces Release of Cytochrome c from Mitochondria Isolated from Human Neuroblastoma Cells

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Abstract

Paclitaxel is an antimicrotubule agent that induces mitotic block and apoptosis. We show for the first time that paclitaxel acts directly on mitochondria isolated from human cancer cells. In isolated yeast mitochondria, paclitaxel (15 μM) induced an 18% increase in the respiration rate, with no concomitant release of cytochrome c. In isolated neuroblastoma mitochondria, paclitaxel (10–100 μM) induced a 27–72% release of cytochrome c. Release was prevented by cyclosporin A, suggesting the involvement of the permeability transition pore. Doxorubicin did not induce cytochrome c release, whereas vinorelbine, another antimicrotubule agent, did. Thus, antimicrotubule agents can directly affect mitochondria to induce apoptosis.

Introduction

For years, the known function of mitochondria was limited to energy production through a process in which electrons are transferred along a series of respiratory enzyme complexes. This electron transfer is coupled to proton translocation on the positive side of the membrane, and the resulting electrochemical gradient is used to synthesize ATP by ATP synthase (1). It has recently been found that mitochondria can also regulate and promote apoptosis by releasing cytochrome c from the mitochondrial intermembrane space into the cell cytosol (2–4). How cytochrome c is released by mitochondria is still unclear (5). The main hypothesis is that the transient or sustained opening of the PTP (3), a multiprotein complex formed at the contact site between the inner and the outer mitochondrial membranes, allows the release of cytochrome c with or without mitochondrial swelling. Cytochrome c release may also occur by changes in the permeability of the mitochondrial outer membrane, partially in response to Bcl-2 family proteins (5, 6). Paclitaxel (Taxol), an anticancer drug that is highly efficacious in the treatment of several malignancies (7), is an antimicrotubule agent that stabilizes the microtubule network and inhibits the dynamics of microtubules (8). Paclitaxel induces apoptosis after G2-M-phase blockage and Bcl-2 phosphorylation, but the mechanisms of paclitaxel-induced apoptosis remain controversial (9). We have previously shown that mitochondrial transition permeability was modified simultaneously with activation of caspase-8 and caspase-3 during apoptosis induced by paclitaxel in proliferating cells (10). Moreover, mitochondria are involved in neuroblastoma cell apoptosis mediated by anticancer agents (11). However, few studies have investigated the direct effects of anticancer agents on isolated mitochondria (12–15). In the present study, we tested the effects of paclitaxel, vinorelbine, and doxorubicin on mitochondria isolated from yeast and human neuroblastoma cancer cells in a cell-free system. We have previously reported that these three drugs induce apoptosis in neuroblastoma SK-N-SH cells (16). Here we present the first evidence that antimicrotubule agents, but not doxorubicin, have a direct effect on mitochondria isolated from both yeast Saccharomyces cerevisiae, in which they induce an increase in respiration, and neuroblastoma cells, in which they induce the release of cytochrome c.

Materials and Methods

Drugs. Stock solutions of paclitaxel (Sigma, St. Louis, MO) and CsA (Sigma) were prepared in DMSO. Stock solutions of vinorelbine (Pierre Fabre Oncologie, Paris, France) and doxorubicin (Dakota, Créteil, France) were prepared in water. Stock solutions were stored at −20°C, with the exception of CsA and vinorelbine solutions, which were stored at 4°C. The highest final DMSO concentration used was 0.2%.

Yeast and Cell Culture. Yeast strain KM-91 was cultured as described previously (17). Human neuroblastoma SK-N-SH cells were cultured in RPMI 1640 (16).

Isolation of Mitochondria. Yeast mitochondria were prepared as described previously (17). Mitochondria were isolated from yeast cells as follows (18). Briefly, 3 × 107 SK-N-SH cells were trypsinized and washed with ice-cold PBS. The cell pellet was suspended in buffer A (250 mM sucrose, 20 mM HEPES, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl2, phenylmethylsulfonyl fluoride, and protease inhibitors) at 4°C. Cells were homogenized with 45 strokes in a glass homogenizer (Kontes, Vineland, NJ) and centrifuged at 800 × g for 10 min to remove unbroken cells and nuclei. Mitochondria were then pelleted by centrifugation at 15,000 × g for 10 min at 4°C. The mitochondrial pellet was rinsed twice in buffer A and immediately resuspended, aliquoted, and incubated with drugs as indicated.

Measurement of RR in Yeast Mitochondria. KM-91 yeast mitochondria (1.5 mg) were suspended in respiration buffer with a range of concentrations of paclitaxel (15–225 μM) and incubated for 40–120 min at 25°C. NADH (3 mM) and succinate (50 mM) were used as substrates to initiate respiration, and the rate was measured using a Clark oxygen electrode (17). The RR equals the slope of oxygen consumption over time.

Incubation of Isolated Mitochondria with Drugs. Neuroblastoma cell mitochondria were incubated for 2 h in buffer A at 37°C with CsA (2 μM), a range of concentrations of paclitaxel (10, 50, and 100 μM), doxorubicin (50 μM), vinorelbine (20 μM), and paclitaxel (50 μM) or vinorelbine (20 μM) after a 2-min preincubation with CsA (2 μM). Isolated yeast mitochondria were incubated (0.5–4 h) with a range of paclitaxel concentrations (20–100 μM) at temperatures ranging from 25°C to 30°C. Equivalent amounts of DMSO were added to control samples.

Western Blot Analysis of Cytochrome c from Mitochondria Isolated from Neuroblastoma Cells. After centrifugation (10 min, 15,000 × g) of treated mitochondria, supernatants were removed carefully. Pellets were lysed in buffer B (2 mM EDTA, 100 mM NaCl, 1 mM orthovanadate, 1% Triton X-100, and 50 mM Tris [pH 7.5]), loaded on a 15% SDS polyacrylamide gel, and transferred to nitrocellulose membranes (19). Membranes were incubated with polyclonal antibodies against cytochrome c (20). After washing, membranes were incubated with the secondary antibody conjugated with horseradish peroxidase. The bands were visualized with an enhanced chemiluminescence reagent (21).
transferred to a nitrocellulose membrane, and incubated with cytochrome c monoclonal antibody (7b82C12; PharMingen, San Diego, CA) and an antimouse monoclonal antibody conjugated with peroxidase. Visualization and densitometric quantitation were performed using enhanced chemiluminescence (Amersham, Aylesbury, United Kingdom) and Traitima, an in-house densitometric software in Visual BASIC for Windows 95 (19). Briefly, the gel images were digitized, and the gray level of the pixels was used to calculate the area of each spot and to plot the intensity curves. A monoclonal antibody against VDAC (529534; Calbiochem, La Jolla, CA), a major component of mitochondrial membranes (20), was used to ensure that equal amounts of mitochondrial proteins were loaded onto the gel and that no mitochondrial protein remained in the supernatant. α-Tubulin antibody (N356; Amersham) was used to demonstrate the presence of tubulin in mitochondrial membranes and the absence of contamination of mitochondrial preparations.

Transmission Electron Microscopy. Aliquots of isolated mitochondria were treated with 100 μM paclitaxel or 0.2% DMSO for 2 h at 37°C, fixed in 4% glutaraldehyde, dehydrated in ethanol, embedded in Epon, and cut into thin sections (19). The samples were imaged by a transmission electron microscope (JEOL 100C). The longest diameter of all mitochondria in a given area was measured on printed photographs. Statistical analysis was performed using Student’s t test.

Results

Paclitaxel Induced an Increase in the RR of Mitochondria Isolated from Yeast. Paclitaxel (≥15 μM) induced a significant increase (18 ± 9%) in the RR of isolated yeast mitochondria (P < 0.001; Fig. 1A). No increase was observed at lower paclitaxel concentrations, and increasing the paclitaxel concentration to a level as high as 225 μM induced no greater effect (Fig. 1B). A minimum of 0.5 h of incubation was required to observe the increase. Paclitaxel induced the same increase regardless of whether NADH, succinate (substrate for complex I and II of the respiration chain, respectively), or both were tested. The uncoupler carbonyl cyanide m-chlorophenylhydrazone (25 μM) induced an additional increase in the RR even after paclitaxel treatment (data not shown). Thus, paclitaxel increased the RR by acting directly on isolated yeast mitochondria.

Paclitaxel Did Not Induce Cytochrome c Release from Yeast Mitochondria. Because paclitaxel had a direct effect on respiration in isolated yeast mitochondria, we investigated whether it could also induce the release of cytochrome c. We found no significant release of cytochrome c (9.1%, 9.2%, and 8.6%, respectively, for control or mitochondria incubated with paclitaxel for 2 and 4 h; Fig. 1C) by Western blot analysis of cytochrome c in the supernatants of centrifuged yeast mitochondria that had been incubated with paclitaxel (20–100 μM) for 2–4 h. Increasing the temperature from 25°C to 30°C did not modify these results (data not shown). Thus, paclitaxel induced an increase in the RR but did not induce cytochrome c release from the yeast mitochondria.

Paclitaxel Induced the Release of Cytochrome c from Mitochondria Isolated from Neuroblastoma Cells. Mitochondria were successfully isolated from SK-N-SH cells as measured by Western blots of VDAC, an outer mitochondrial membrane protein, and tubulin (Fig. 2A). VDAC was present at the same level in the mitochondrial pellets but was absent in the supernatants of all of the samples. The mitochondrial pellets contained a significant amount of tubulin (Fig. 2A right panel) that appears to be in the mitochondria and not simply a contaminant of the pellets. Pelleted tubulin did not appear to be a contaminant for several reasons. First, it did not appear in the supernatants and thus is not soluble. Secondly, microtubules were not pelleted under the conditions for isolating mitochondria (4°C, 15,000 × g, 10 min) because no microtubules were observed by transmission electron microscopy of the paclitaxel-treated samples (data not shown). Thus, immunodetection of tubulin in mitochondrial pellets confirms the presence of tubulin in mitochondria (21).

Paclitaxel induced the release of cytochrome c from neuroblastoma mitochondria in a concentration-dependent manner (Fig. 2A), whereas no release occurred in a control experiment with 0.2% DMSO. Quantitation of Western blots of mitochondrial pellets and supernatants indicated that paclitaxel (10–100 μM) induced a concentration-dependent release of cytochrome c (27 ± 8% to 72 ± 9% of total mitochondrial cytochrome c, respectively; Fig. 2B).

To examine how cytochrome c was released, we pretreated mitochondria isolated from neuroblastoma cells with CsA, a compound known to inhibit PTP opening. After preincubation with CsA, the release of cytochrome c was inhibited (Fig. 2A). Thus, paclitaxel induced the release of cytochrome c from mitochondria isolated from SK-N-SH neuroblastoma cells in a CsA-sensitive manner, suggesting the involvement of PTP.

Effects of Doxorubicin and Vinorelbine on Cytochrome c Release from Mitochondria Isolated from Neuroblastoma Cells. Because paclitaxel induced translocation of cytochrome c by acting directly on mitochondria, we investigated whether this novel property was shared by other antimicrotubule agents or doxorubicin, a drug whose mechanism of action is independent of microtubules. Interestingly, doxorubicin (50 μM) had no effect on the cytochrome c release...
from neuroblastoma mitochondria, but vinorelbine (20 μM), an antimicrotubule agent that depolymerizes microtubules, did induce cytochrome c release from mitochondria in a CsA-sensitive way (Fig. 2C).

**Mitochondrial Swelling after Paclitaxel Treatment in Vitro.**

Swelling is an important feature of apoptotic mitochondria (5). Thus, we wanted to know whether the PTP-dependent release of cytochrome c from neuroblastoma mitochondria induced by paclitaxel was associated with mitochondrial swelling. Mitochondria isolated from neuroblastoma cells were treated with paclitaxel under the conditions that induced cytochrome c release and were examined by transmission electron microscopy (Fig. 3, A and B). The mean diameter of paclitaxel-treated mitochondria (1.2 ± 0.05 μm) was significantly greater than that of control mitochondria (1.0 ± 0.04 μm; P < 0.05; Fig. 3C), suggesting that the PTP opening resulted in significant swelling and thus was sustained and not merely transient.

**Discussion**

In the present study, we show for the first time that paclitaxel acts directly on isolated mitochondria by increasing the RR in mitochondria isolated from yeast and by inducing the translocation of cytochrome c from mitochondria isolated from human cancer cells in a PTP-dependent manner. The association with mitochondrial swelling suggests a sustained opening of PTP. An increase in respiration can be attributed to either a direct effect of paclitaxel on the complexes of the mitochondrial respiration chain or a partial uncoupling effect. Our results show that (a) paclitaxel does not affect complex I or II of the respiration chain; (b) paclitaxel does not induce a nonspecific uncoupling of mitochondria, as does carbonyl cyanide m-chlorophenylhydrazone; and (c) paclitaxel induces the opening of PTP. Together, these results suggest that the increase in the RR may be related to a possible partial uncoupling of the respiratory chain due to opening of the PTP by paclitaxel.

Paclitaxel concentrations required to induce the release of cytochrome c from isolated mitochondria are higher than those usually used to induce apoptosis in cultured cells. However, paclitaxel accumulates in cells. For example, Jordan et al. (22) found that addition of 10 or 100 nM paclitaxel to HeLa cells resulted in an intracellular paclitaxel concentration of 4.8 and 40.5 μM, respectively. Thus, the intracellular concentration of paclitaxel can reach 500 times the extracellular concentration. Because 1 μM paclitaxel is necessary to
Two additional antitumor drugs were tested, vinorelbine and doxorubicin. Interestingly, vinorelbine, a depolymerizing antimicrotubule agent, also induced the release of cytochrome c in a CsA-sensitive manner, whereas doxorubicin, whose mechanism of action is independent of microtubules, had no effect at a concentration of 50 μM. Doxorubicin did not release cytochrome c even at concentrations 100 times higher than the extracellular concentration inducing apoptosis in SK-N-SH cells (16). Paclitaxel and vinorelbine both bind to tubulin in microtubules but bind to different sites on the tubulin molecule (8). Thus the direct mitochondrial effect of paclitaxel and vinorelbine may be a phenomenon shared by many antimicrotubule agents. Moreover, evidence suggests that lonidamine and arsenic, compounds that can induce apoptosis by acting directly on mitochondria, may also act on the microtubule network (26, 27). Thus, antimicrotubule and antimitochondrial agents may be able to perturb mitochondrial and microtubule physiology as a result of a common target or a common intermediate actor and thus cooperate to induce apoptosis. Bim, a BH3-only protein proapoptotic member of Bcl-2 family (28), calcium (29), and tubulin (25) are possible candidates for effectors of the microtubule-mitochondria interrelationship during apoptosis.

How paclitaxel induces the opening of PTP is not known. Our results confirm that tubulin is present on mitochondrial membranes as reported previously (21), but it is not yet known whether the tubulin is polymerized (which would enhance the binding of paclitaxel to mitochondria). Nevertheless, when present in the mitochondrial membranes, tubulin could adopt a conformation similar to its conformation in the microtubule. Paclitaxel may also interact with Bcl-2 (30) to mediate changes in PTP. The real impact of the direct mitochondrial effect of paclitaxel on apoptosis must be further investigated. The release of cytochrome c in a cell-free system as a result of a direct effect of paclitaxel on mitochondria might explain how paclitaxel could induce apoptosis without mitosis and phosphorylation of Bcl-2 (9).

Finally, we have demonstrated that antimicrotubule agents are the first widely used family of anticancer agents that act directly on mitochondria. Such an effect has also been reported with some new anticancer agents (lonidamine, arsenite compounds, retinoic acids, and betulinic acid; Refs. 12–15). Altogether, these data suggest that (a) mitochondria may be a new target for anticancer agents, and thus antimitochondrial agents may represent a new class of anticancer agents; and (b) antimicrotubule agents may work at least in part because of their antimitochondrial effect.

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References


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