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Original Contribution

The superoxide dismutase inhibitor diethyldithiocarbamate has antagonistic effects on apoptosis by triggering both cytochrome *c* release and caspase inhibition

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Abstract

Tumor necrosis factor-α (TNF-α) and etoposide both trigger a large and rapid production of reactive oxygen species (ROS) in HeLa cells. This occurs before translocations of the proapoptotic Bax and cytochrome *c* proteins, the loss of mitochondrial membrane potential (ΔΨm), and apoptosis. We have used diethyldithiocarbamate (DDC), a well-known inhibitor of Cu, Zn superoxide dismutase to study the role of ROS in this system. We report that DDC strongly inhibits caspase activation, loss of ΔΨm, and cell death induced by TNF-α or etoposide. Surprisingly, DDC does not inhibit Bax and cytochrome *c* translocations. On the contrary, we have observed that DDC can trigger the translocations of these proteins by itself, without altering ΔΨm. Here, we report that DDC has at least two antagonistic apoptosis regulation functions. First, DDC triggers ROS-dependent Bax and cytochrome *c* translocations, which are potentially proapoptotic, and second, DDC inhibits caspase activation and activity, loss of ΔΨm, and cell death, in a ROS-independent manner. Our results suggest an interesting model in which ROS-dependent Bax and cytochrome *c* translocations can be studied without interference from later apoptotic events.

Keywords: Apoptosis; Mitochondria; Reactive oxygen species; Cytochrome *c*; Bax; Caspases; Superoxide; Superoxide dismutase; DDC; Free radicals

Introduction

Reactive oxygen species (ROS) are a family of highly reactive molecules that include hydroxyl radical ($HO^{\bullet}$), superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and organic peroxide radicals. ROS can be produced in vivo by many enzymes systems, such as NADPH oxidase, lipoxygenases, cyclooxygenases, and cytochrome P450. Under physiological conditions, the mitochondrial respiratory chain is the major site for ROS production in cells [1]. Electrons carried by the electron transport chain can be diverted from the pathway to oxygen, generating superoxide anion. ROS are extremely transient species due to their high chemical reactivity. It is this chemical reactivity that is responsible for their destructiveness on DNA, proteins, carbohydrates, and lipids. However, under normal conditions, antioxidants (reduced glutathione (GSH), catalase, superoxide dismutase (SOD)) usually prevent tissue damage [2]. When ROS overcome the defense systems of the cell, there is an alteration of the redox homeostasis, leading to oxidative stress. As well as their destructiveness, it has been suggested that ROS may also act as chemical messengers. For instance, ROS appear to be involved in receptor-mediated pathways and transcriptional activation [3].

Abbreviations: BHA, butylated hydroxyanisole; BSA, bovine serum albumin; Cyt *c*, cytochrome *c*; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DDC, diethyldithiocarbamate; DDT, dithiotreitol; DFO, deferoxamine; $DiOC_6(3)$, 3,3′-diethyloxacarbocyanine; DISC, death-inducing signaling complex; E, emetine; Eto, etoposide; FDA, fluorescein diacetate; NBT, nitroblue tetrazolium; PARP, poly(ADP ribose) polymerase; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF, tumor necrosis factor; XOD, xanthine oxidase; zVAD, z-Val-Ala-DL-Asp-fluoromethylketone.

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Necrotic cell death due to oxidative stress is well documented [4]. This phenomenon implies massive cellular damage, which would mostly be caused by the highly reactive hydroxyl radical. However, the possibility of ROS being signaling molecules in more physiological deaths, such as apoptosis, is a more recent idea. Since the discovery of the contribution of ROS to the TNF-α (tumor necrosis factor-α)-induced cytotoxicity [5,6], there is growing evidence that these compounds may play a central role in cell-death transduction pathways. Indeed, there are several observations suggesting that ROS may mediate apoptosis: (i) the addition of ROS or the depletion of endogenous antioxidants can promote apoptosis [7–9], (ii) apoptosis can sometimes be delayed or inhibited by antioxidants [10–12], and (iii) many apoptotic stimuli, such as TNF-α, glucocorticoids, growth factor withdrawal, and human immunodeficiency virus infection, have been shown to stimulate ROS production [13–16].

Apoptosis is mediated by a family of cystein proteases known as caspases. These are initially expressed in cells as inactive zymogens. When initiator caspases, such as caspase-8 and caspase-9, are activated by oligomerization, they cleave the precursor forms of effector caspases, such as caspase-3, -6, or -7, which then mediate cell death [17]. In mammals, there are two main pathways by which caspase activation is triggered: the intrinsic and extrinsic apoptotic pathways. The intrinsic pathway is triggered by various extracellular and intracellular signals such as genotoxic or oxidative stresses. These signals are mainly transduced to the mitochondria, which then undergo a series of biochemical events resulting in the permeabilization of the outer mitochondrial membrane [18]. Among other proapoptotic molecules, cytochrome *c* (cyt c) is released from the mitochondrial intermembrane space and induces the formation of the apoptosome complex, which recruits caspase-9 and promotes caspase activation [19]. The extrinsic pathway is activated by the binding of ligands, such as FasL or TNF-α, to their receptors on the cell surface [20,21]. This induces the formation of the death-inducing signaling complex (DISC), which recruits caspase-8 and promotes a cascade of procaspase activation [22]. This pathway may also require the involvement of mitochondria, notably through the caspase-8-dependent production of the proapoptotic protein tBid [23,24]. The mitochondrial pathway is regulated by members of the Bcl-2 family, which includes both antiapoptotic proteins (such as Bcl-2) and proapoptotic proteins (such as Bax). These proteins repress or stimulate apoptosis by forming homodimers and heterodimers with each other and by controlling the permeability of the outer mitochondrial membrane.

The reasons for the increased ROS generation and the molecular mechanism underlying ROS signaling in cell-death transduction pathways are far from being completely understood. In most ROS-dependent cell-death systems, ROS accumulation has been shown to require a functional mitochondrial respiratory chain [13,25–27]. Disturbances in the electron flow causes the accumulation of ROS in the mitochondria, but the origin of these disturbances is still unclear [1]. A direct influence of ROS on the apoptotic process may be related to a general damaging effect on mitochondria, where these molecules are produced, which could provoke the release of cyt *c*. An alternative explanation may be that increased ROS production causes a more specific effect; proapoptotic members of the Bcl-2 family may be targets for such a regulatory mechanism. In healthy cells, proteins such as Bax are cytosolic, whereas upon death, they redistribute to mitochondria, promoting cell death.

We have previously shown in HeLa cells that TNF-α-induced apoptosis involves early mitochondrial ROS production that strongly accelerates the process of cell death [13]. We studied the role of a range of drugs having prooxidant or antioxidant activities to understand better the role of these ROS in the apoptotic process. In particular, we have used a well-known inhibitor of Cu, Zn SOD, called diethyldithiocarbamate (DDC). This is a thiol-containing molecule and a potent metal ion-chelating agent [28,29]. The SODs catalyze the conversion of the superoxide anion into hydrogen peroxide, which can then be detoxified by several other enzymes, such as catalase and glutathione peroxidase [2]. DDC has been described both to increase superoxide concentration and to inhibit the detoxification of ROS [30–32].

Etoposide is a topoisomerase II inhibitor, which triggers the intrinsic caspase activation pathway, involving mitochondrial perturbations [33], whereas TNF-α is a cytokine that triggers the extrinsic caspase activation pathway but does not necessarily involve the mitochondria [21]. We show that both apoptotic processes are dependent on a high-level production of ROS, which is then followed by mitochondrial perturbations, such as translocations of Bax and cyt *c* and a loss of mitochondrial membrane potential ($\Delta\Psi m$). Here, we have tested the role of DDC on HeLa cells induced to undergo apoptosis by the addition of TNF-α or etoposide. We show that, after the addition of TNF-α or etoposide, DDC strongly inhibits the reduction in $\Delta\Psi m$, caspase activation, and cell viability, demonstrating that DDC has antiapoptotic properties. However, we have observed that DDC does not inhibit the release of cyt c, suggesting a model in which cyt *c* is cytosolic but caspases remain inactive and cells remain alive. Interestingly, we have shown that DDC by itself triggers the translocations of Bax and cyt *c* in the absence of TNF-α or etoposide. We have also shown that the action of DDC on Bax and cyt *c* is ROS dependent and is modulated by several antioxidants, whereas the action of DDC on caspase and cell viability is modulated by thiol reducing agents such as dithiotreitol (DTT). This suggests that DDC may directly inhibit caspases by forming disulfides links with them, as previously shown for disulfiram, another member of the dithiocarbamate family [34,35]. We also show that DDC inhibits the processing of the initiator caspase-9 and PARP cleavage (a target of caspase-3), suggesting that DDC may alter both caspase activation and activity.

In conclusion, we show that DDC should be used cautiously to study the role of ROS during apoptosis, as it appears to have several separate ROS-dependent and -independent activities. Furthermore, our results also suggest an interesting model in which the ROS-dependent and early events leading to Bax and cyt *c* translocations may be studied without interference from later apoptotic events.

Materials and methods

Reagents

Arachidonyl trifluoromethyl ketone (AACOCF3), butylated hydroxyanisole (BHA), catalase, deferoxamine (DFO), diethyldithiocarbamate (DDC), diphenyleneiodonium chloride (DPI), dithiothreitol, emetine (E), indomethacin, rotenone, sodium azide (NaN_3), tiron, tumor necrosis factor-α, and etoposide (eto) were from Sigma. 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) and 3,3′-diethyloxacarbocyanine ($DiOC_6(3)$) were from Molecular Probes. z-Val-Ala-DL-Asp-fluoromethylketone (zVAD) was from Bachem. $CuSO_4$ and $MgSO_4$ were from Merck. Stock solutions of BHA (200 mM), DCFH-DA (20 mM), $DiOC_6(3)$ (1 mM), and indomethacin (5 mM) were prepared in ethanol. zVAD (50 mM) was prepared in methanol. DDC (100 mM), DFO (10 mM), DTT (1 M), tiron (1 M), NaN_3 (1 M), $CuSO_4$ (100 mM), and $MgSO_4$ (100 mM) were prepared in water. Catalase (10^5 U/ml), emetine (1 mg/ml), and TNF-α (1 μg/ml) were prepared in serum-free medium. AACOCF3 (10 mM), DPI (3 mM), and rotenone (25 mM) were prepared in dimethyl sulfoxide (DMSO). All stock solutions were stored at –20°C.

Cell line, cell culture and induction of apoptosis

HeLa cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM/F12) supplemented with 10% fetal bovine serum together with penicillin (100 μg/ml), streptomycin (100 U/ml), and glutamax (1% v/v) from Invitrogen. Cell death was induced by the addition on exponentially growing adherent cells of etoposide (20 ng/ml) or by the addition of TNF-α (2.5 ng/ml) plus emetine (1 μg/ml). After incubation for 16 h, cells were analyzed by flow cytometry or immunofluorescence.

Flow cytometry

Flow cytometric measurements were performed using a XL3C flow cytometer (Beckman-Coulter). Fluorescence was induced by the blue line of an argon ion laser (488 nm) at 15 mW. Green fluorescence was collected with a 525-nm band pass filter. Analyses were performed on 10^4 cells and data were stored in "listmode." The mitochondrial membrane potential ($\Delta\Psi m$) was assessed by the retention of $DiOC_6(3)$. This cationic lipophilic fluorochrome is a cell-permeable marker that, at low doses, specifically accumulates in the mitochondria depending on the mitochondrial membrane potential [36]. DCFH-DA is a fluorogenic freely permeable tracer, which is deacylated by intracellular esterases to the nonfluorescent compound DCFH and oxidized to the fluorescent compound DCF by a variety of peroxides, including hydrogen peroxide [37]. Cell viability was assessed with the nonfluorescent FDA (fluorescein diacetate), which becomes fluorescent (free fluorescein) when cleaved by esterases in living cells [38]. As esterases participate in the general cell metabolism, cells can be considered metabolically dead when they no longer produce free fluorescein. The activation of caspases was followed with the CaspaTag kit (Intergen), which uses a peptide (Val-Ala-Glu) coupled to a fluorochrome that specifically and irreversibly associates with activated caspases in the cell.

After drug treatment, the media from the culture dishes (containing late apoptotic cells) were kept in centrifuge tubes. The adherent cells (containing living and early apoptotic cells) were detached using trypsine, pooled with the corresponding media, centrifuged, and resuspended in complete medium at a concentration of 1×10^6 cells/ml. Cells were then loaded with 0.1 μM $DiOC_6(3)$, 20 μM DCFH-DA, or 2 μg/ml FDA by incubation for 30 min at 37°C, or tested with the CaspaTag kit.

Statistical analysis

Statistical analysis was determined using Student's t test. $P < 0.05$ was accepted as significant.

Immunofluorescence

Cells were grown on glass coverslips. To follow Bax and cyt c translocations, cells were fixed with 3.7% paraformaldehyde for 15 min at room temperature and permeabilized with acetone for 1 min at −20°C. Cells were incubated for 1 h with the 1/200 rabbit anti-Bax (N-20, Santa Cruz Biotechnology) and 1/200 mouse anti-cyt c (BD Biosciences) primary antibodies in PBS-BSA 1%, washed with PBS, and then incubated with 1/100 Rhodamine-conjugated anti-mouse Ig and 1/100 FITC-conjugated anti-rabbit Ig (Jackson ImmunoResearch Laboratories). Nuclei were stained by incubation with Hoechst 33342 (1 μg/ml) for 5 min, and cells were washed with PBS. Coverslips were mounted in a glycerol/PBS solution (Citifluor AF1) and cells were examined by epifluorescence under a DMR Leica microscope and photographed. We counted about 500 cells for each point to evaluate the percentage of cells with relocalized Bax, cyt c, or fragmented nuclei.

Superoxide dismutase activity

The superoxide dismutase activity in HeLa cell lysates was determined by using the superoxide dismutase kit from R&D Systems according to the manufacturer's instructions. In the assay, superoxide anions generated by xanthine oxidase (XOD) convert nitroblue tetrazolium (NBT) to NBT-diformazan which absorbs light at 550 nm. SOD reduces the superoxide anion concentration and thereby lowers the rate of NBT-diformazan formation. Briefly, cells were detached with trypsine after treatment and washed once with cold PBS. The cell pellet was suspended in cell lysis solution at 5×10^6 cells/ml. Cell lysates were centrifuged at 14,000g for 5 min at 4°C and supernatants were kept on ice. For each assay, 500 μl of the supernatants was mixed with xanthine, NBT, and XOD solutions and absorbance at 550 nm was measured 5 min after the beginning of reaction. We determined the 100% of SOD activity by measuring the difference between the absorbances of HeLa control cells and a negative control without cell lysate.

Western blot analysis

After harvesting cells with a scraper, and washing with PBS, cells were suspended in lysis buffer (50 mM Tris pH 6.8, 2% SDS, 50 mM DTT). The protein concentration was determined using the Bio-Rad protein assay. A total of 50 μg/well of the boiled samples was loaded onto a 4–12% NuPAGE gel (Invitrogen). After migration, the proteins were electrotransferred onto a PVDF membrane (Millipore). The membranes were blocked with TBS-Tween and 5% fat milk by incubation for 1 h, and proteins were incubated overnight with specific antibodies: mouse and rat anti-caspase 9 (5B4, Immunotech and No. 9506, Cell Signaling Technology) and mouse anti-PARP (C2-10, R&D systems). After washing in TBS-Tween, the membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). The proteins were visualized using the ECL detection kit (Amersham).

Results

Diethyldithiocarbamate is an inhibitor of the extrinsic and the intrinsic pathways of apoptosis in HeLa cells

We have previously shown that the extrinsic pathway of apoptosis, induced by TNF-α and emetine in HeLa cells, involves an early production of ROS in the mitochondria, which accelerates cell death [13,39]. We used emetine because the interaction between TNF-α and its receptors triggers antagonistic signals: the activation of caspases through the DISC formation and the expression of antiapoptotic proteins through the activation of NF-κB [39]. Emetine is an inhibitor of protein synthesis which prevents the NF-κB-dependent inhibition of apoptosis without affecting the DISC formation, therefore potentiating the effect of TNF-α. We have also used etoposide, an inhibitor of topoisomerase II, to induce the intrinsic pathway of apoptosis [33]. We tested several aspects of the apoptotic process: caspase activation, cell viability loss, ROS production, and loss of mitochondrial membrane potential (ΔΨm) (see Materials and methods). We found that both emetine/TNF-α (E/TNF) and etoposide (eto) induced caspase activation (CaspaTag) and cell death (FDA) in HeLa cells (Fig. 1). Furthermore, apoptosis triggered by either E/TNF or etoposide induced an increase in ROS (DCFH-DA) production and a drop in ΔΨm ($DiOC_6(3)$). The reduction in ΔΨm suggested that mitochondria were involved in the apoptotic process, as the release of several mitochondrial proapoptotic proteins, such as cyt *c*, is

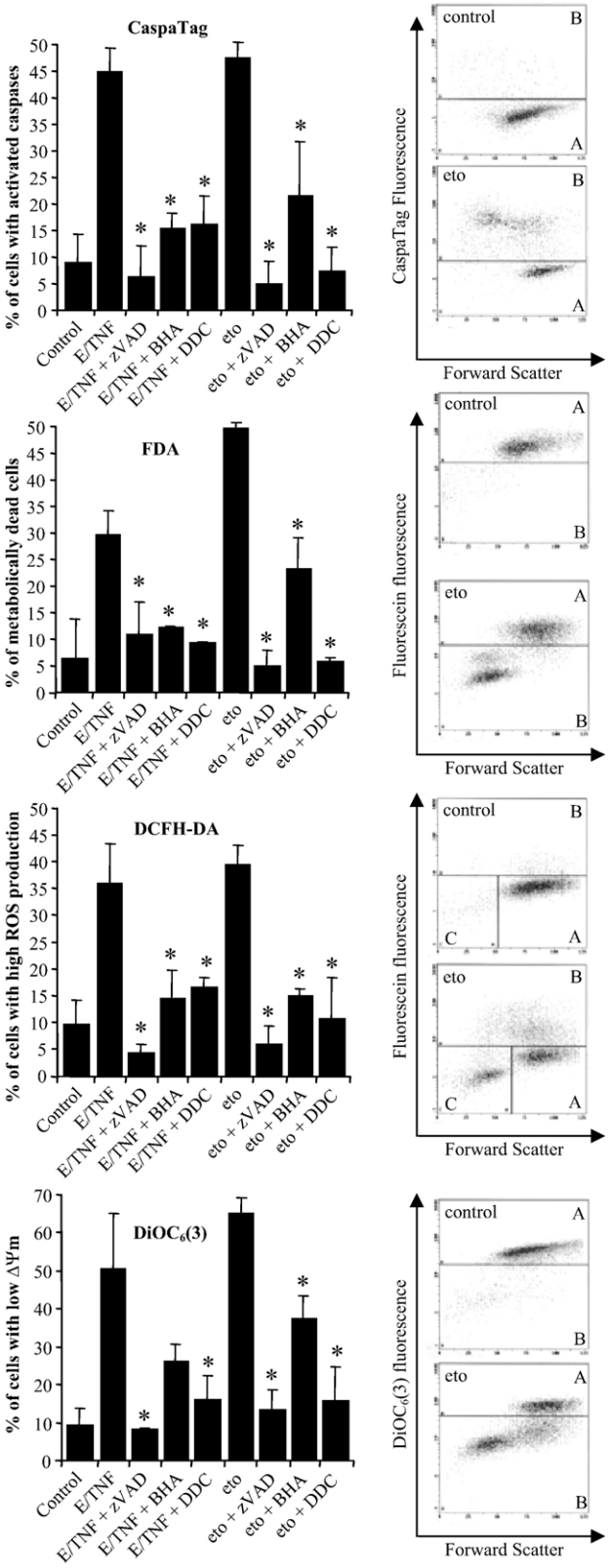

Fig. 1. Protective effects of zVAD, BHA, and DDC on apoptosis induced by TNF-α and emetine (E/TNF) or etoposide (eto). Left panel, data from cytometric analysis, indicating the percentage of cells: with activated caspase (CaspaTag), metabolically dead (FDA), having high ROS production (DCFH-DA), or having low ΔΨm ($DiOC_6(3)$). Right panel, typical cytograms (forward scatter (FSC) versus fluorescence) obtained from flow cytometric analysis of HeLa cells incubated for 16 h with etoposide (eto) or not (control). For all types of labeling, windows A and B represent living cells and apoptotic cells, respectively. Percentages indicated on left panel were measured from the corresponding B windows. For DCFH-DA, window C corresponds to late apoptotic cells that have lost their plasma membrane integrity and cannot retain the oxidized DCF. The average values and standard deviations were calculated from three independent experiments. *Statistically significant difference of cells treated with different drugs versus cells treated with E/TNF or etoposide alone. Statistical analysis was determined using Student's *t* test. $P < 0.05$ was accepted as significant. For E/TNF + BHA with $DiOC_6(3)$, $P = 0.054$.

often accompanied by a permeabilization of the outer mitochondrial membrane [40,41]. Next, we studied the role of three drugs—zVAD-fmk, butylated hydroxyanisole, and diethyldithiocarbamate-to characterize better the apoptotic process induced by E/TNF or etoposide in HeLa cells.

zVAD is a broad-spectrum caspase inhibitor and inhibits apoptosis in most systems studied. We found that zVAD (50 μM) inhibited caspase activation and cell death (Fig. 1), showing that E/TNF and etoposide trigger a caspase-dependent apoptotic cell death in HeLa cells. zVAD also inhibited the increased production of ROS and the loss of $\Delta\Psi m$, showing that both events occur downstream from caspase activation.

The antioxidant butylated hydroxyanisole is an inhibitor of the mitochondrial respiratory chain [42,43]. We found that BHA (100 μM) reduced the percentage of cells overproducing ROS (DCFH-DA) as well as caspase activation (CaspaTag) and cell death (FDA) (Fig. 1), showing that ROS are accelerators of apoptosis in this system.

We next tested the role of the Cu, Zn SOD inhibitor diethyldithiocarbamate [28,44]. Initially, we aimed to characterize the ROS that trigger apoptosis. Several have observed that $O_2^{\bullet-}$ and H_2O_2 may have antagonistic effects on the regulation of apoptosis, with H_2O_2 being proapoptotic and $O_2^{\bullet-}$ being antiapoptotic [45–47] and it has been shown that DDC can simultaneously decrease the level of H_2O_2 and increase the level of $O_2^{\bullet-}$ [30–32,45]. Here, we found that DDC (500 μM) strongly inhibited caspase activation, cell death, ROS production, and the loss of $\Delta\Psi m$ (Fig. 1). As DCFH preferentially reacts with peroxides and DDC inhibits apoptosis, we initially thought that DDC would diminish the level of H_2O_2 and that the corresponding accumulation of $O_2^{\bullet-}$ would inhibit apoptosis.

We also studied the translocations of proapoptotic proteins, such as Bax and cyt *c*. Some proapoptotic proteins of the Bcl-2 family, such as Bax, translocate to the mitochondria and participate in the release of cyt *c* into the cytosol in the intrinsic apoptotic pathway [18,48]. We examined the intracellular location of Bax and cyt *c* as well as the morphology of the nuclei, indicating the apoptotic status of the cells. We found that in control cells, nuclei are intact, Bax is cytosolic, and cyt *c* is mitochondrial (Fig. 2A). When we added E/TNF or etoposide, we found that some of the nuclei were condensed, indicating which cells were apoptotic. In these cells, we noticed that Bax was located in the mitochondria, whereas cyt *c* was located in the cytosol. This confirmed the results obtained with $DiOC_6(3)$, which suggested that the mitochondria were implicated in both

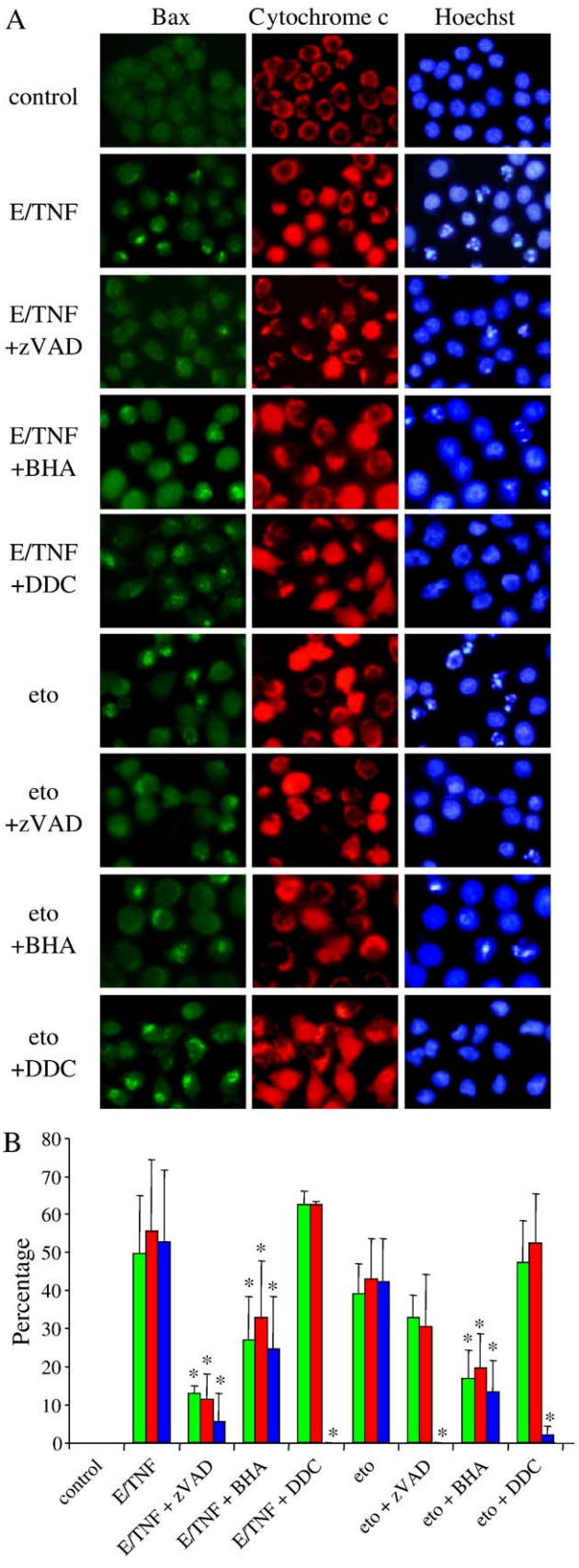

Fig. 2. Effects of zVAD, BHA, and DDC on Bax and cyt *c* translocations and nuclei fragmentation. Immunostaining of Bax and cyt *c* was carried out on HeLa control cells or cells induced to die with E/TNF or etoposide, in the presence or in the absence of zVAD (50 μM), BHA (100 μM), or DDC (500 μM). Nuclei were stained with Hoechst 33342 to characterize the apoptotic cells with fragmented nuclei. Representative pictures of Bax and cyt *c* location and morphology of nuclei are presented in panel A. The average values and standard deviations of cells with mitochondrial Bax (green bars), cytosolic cyt *c* (red bars), and fragmented nuclei (blue bars) were calculated from three independent experiments and are presented in panel B. *Statistically significant difference of cells treated with different drugs versus cells treated with E/TNF or etoposide alone. Two types of labeling can be observed for Bax and cyt *c*: diffuse or spotted, which correspond to cytosolic or mitochondrial distributions, respectively.

E/TNF and etoposide-induced apoptosis. We found that zVAD inhibited nuclear condensation for both types of apoptosis. Bax and cyt *c* translocations were inhibited in the presence of zVAD for E/TNF-induced apoptosis whereas they were not inhibited for etoposide-induced apoptosis. This is consistent with our current understanding of the extrinsic and intrinsic pathways of apoptosis [17]. Indeed, mitochondrial perturbations occur after caspase-8 activation in the extrinsic pathway, whereas Bax and cyt *c* translocations occur before caspase-9 activation in the intrinsic pathway.

We found that BHA inhibited both Bax and cyt *c* translocations and nuclei condensation. As Bax translocation participates in the release of cyt *c* from the mitochondria [49–51], this suggests that ROS may accelerate the apoptotic process in this system by directly or indirectly triggering Bax translocation to the mitochondria. DDC appears to act differently from BHA; the condensation and fragmentation of nuclei is completely inhibited whereas Bax and cyt *c* translocations are not inhibited. Fig. 2B shows the quantification of the results for Fig. 2A.

Together, these results show that the E/TNF and etoposide-induced apoptosis depend on ROS production and involve mitochondrial perturbations. They also show that the SOD inhibitor, DDC, inhibits all aspects of apoptosis we have examined here, except for Bax and cyt *c* translocations.

DDC induces the translocations of the proapoptotic Bax and cytochrome c proteins without triggering apoptosis

We tested the effect of zVAD, BHA, and DDC alone on the location of Bax and cyt *c*. We found that zVAD and BHA do not change the location of these proteins (data not shown), whereas DDC gave more surprising results. We found that control cells contained Bax in the cytosol and cyt *c* in the mitochondria and showed no nuclei fragmentation (Fig. 3A, left panel). According to flow cytometric analyses, they also showed no particular signs of cell death (Fig. 3A, right panel). We found the same results with flow cytometry for cells treated with 500 μM DDC during 16 h (right panel), confirming that DDC is not toxic for the cells. However, some of the cells harbored mitochondrial Bax and cytosolic cyt *c*, indicating that DDC can trigger the translocations of these proteins by itself. However, this was not followed by nuclear fragmentation, which is consistent with the lack of DDC toxicity. This shows that despite this behavior of Bax and cyt *c* in the presence of DDC the cells are unable to complete apoptosis. Therefore, DDC may have both pro- and antiapoptotic functions: DDC would inhibit apoptosis downstream from its own induction of cyt *c* release.

In order to test this hypothesis, we have removed DDC from cells after 16 h of incubation, when Bax and cyt *c* would already be translocated, and observed the cell phenotype 8 h after rinsing out DDC (i.e., 24 h after the beginning of the experiment, Fig. 3A). We found that the control cells after 24 h of incubation with DDC (+DDC 24 h) were similar to those observed after 16 h of incubation with DDC (+DDC 16 h). For cells that were incubated for 16 h with DDC and then 8 h without DDC (DDC +/− 16 h/8 h), we found that cells harboring translocated Bax and cyt *c* also had fragmented nuclei, which is characteristic of apoptotic cells. Flow cytometry confirmed that the cells could undergo apoptosis after rinsing out DDC, with about 30% of the cells being CaspaTag (VAD) positive and FDA and $DiOC_6(3)$ negative. DCF labeling showed that these cells did not overproduce ROS during apoptosis. These results confirm that the DDC-induced translocations of Bax and cyt *c* have potential proapoptotic properties and that DDC inhibits apoptosis downstream from cyt *c* release.

We determined the percentage of cells with relocated Bax, cytosolic cyt *c*, and fragmented nuclei as a function of DDC concentration (Fig. 3B). From 10 to 50 μM DDC, both the translocations of Bax and cyt c and the nuclear fragmentation were triggered in a small number of cells. At these concentrations, DDC may induce translocations of Bax and cyt *c* but be unable to inhibit caspase activation and apoptosis. Above 50 μM DDC, the percentage of cells with translocated Bax and cyt *c* increased and nuclear fragmentation was no longer seen, showing that both activities of DDC were present. We never observed more than 50% of cells having translocated Bax and cyt *c*, suggesting that a particular susceptibility to DDC may be needed for the cells to respond. This difference may, for example, depend on their antioxidant defenses. Longer incubation times with DDC did not change these results (data not shown).

DDC exerts both pro- and antiapoptotic functions on HeLa cells, respectively ROS-dependent and independent

As DDC can have two simultaneous antagonistic effects on cell death in HeLa cells, we examined whether these antagonistic effects occur over the same DDC concentration range (see Fig. 4). Various concentrations of DDC appear to have similar effects on E/TNF and etoposide-induced apoptosis. We found a partial inhibition of caspase activity, cell death, ROS production, and loss of $\Delta\Psi m$ at concentration between 10 and 100 μM DDC and total inhibition from 250 μM to 1 mM. Thus, the proapoptotic function of DDC appears greatest between 250 and 500 μM (Fig. 3B) and the antiapoptotic function of DDC appears greatest between 250 μM and 1 mM (Fig. 4). That both DDC effects were greatest in the same concentration range (250 to 500 μM) did not exclude that both effects could be due to the same upstream events, triggered by DDC.

We used several drugs that inhibit intracellular ROS production, and several antioxidant and prooxidant molecules or ROS scavengers to determine whether both DDC effects were ROS dependent or not. We first tested these drugs on the ability of DDC to trigger Bax and cyt *c* translocations, in the absence of E/TNF or etoposide. We measured the percentage of cells presenting translocated Bax and cyt *c* after 16 h of treatment with DDC (Table 1). The drugs were systematically incubated with 500 μM DDC. We found that the drugs had no stimulating effects on Bax and cyt *c* translocations (data not shown). As DDC is an inhibitor of Cu, Zn SOD and increases the level of the superoxide anion versus hydrogen peroxide, we

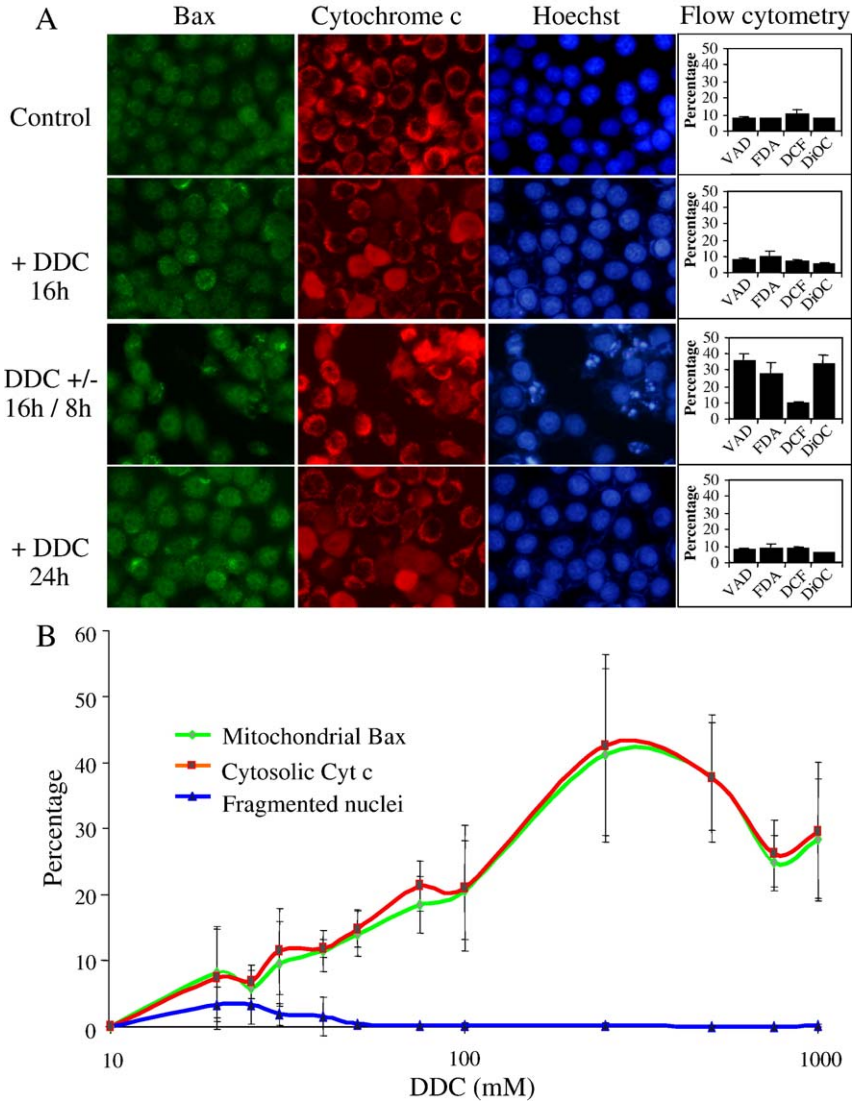

Fig. 3. Proapoptotic property of DDC-induced Bax and cyt *c* translocations. Immunostaining of Bax and cyt *c* was carried out on HeLa cells incubated with or without DDC. Nuclei were stained with Hoechst 33342 to follow nuclear fragmentation. (A) Representative pictures of Bax and cyt *c* location and morphology of nuclei are presented (left panel). The average percentages and standard deviations of flow cytometric analyses (right panel) are also presented for cells: with activated caspases (CaspaTag: VAD positive cells), metabolically dead (FDA negative), having high ROS production (DCF positive), and having low $\Delta\Psi m$ (DiOC negative). The four conditions tested are cells without DDC (control), cells with 500 μM DDC incubated for 16 h (+ DDC 16 h), cells with 500 μM DDC incubated for 16 h and then washed and incubated without DDC for 8 h (DDC +/− 16h / 8h), and cells incubated with DDC 500 μM for 24 h (+ DDC 24h). (B) Influence of DDC concentration on Bax and cyt *c* translocations and nuclei fragmentation, observed after 16 h incubation with variable concentrations of DDC (10 to 1000 μM). Reported data are the average percentages and standard deviations of two (A) or at least four independent experiments (B).

tested the effect of tiron, a scavenger of the superoxide anion [52]. We found that tiron completely inhibited the DDC-induced Bax and cyt *c* translocations, suggesting that superoxide is involved in this effect of DDC.

We then tested the effect of rotenone and NaN_3, two inhibitors of the mitochondrial respiratory chain—complex I and complex IV, respectively. The ubiquinone site in complex III is known to be the major site of mitochondrial ROS production, by converting molecular oxygen to the superoxide anion, which can form other potent ROS. It has been shown that upstream inhibition, acting on complex I, prevents ROS accumulation [6,26], whereas downstream inhibition of the mitochondrial respiratory chain enhances this accumulation [53]. We found that rotenone inhibited the DDC-induced Bax and cyt *c* translocations, suggesting involvement of the mitochondrial respiratory chain, whereas NaN_3 did not have a significant effect (Table 1).

We also tested inhibitors of other nonmitochondrial systems of ROS production, such as NADPH oxidase (DPI) [54], cyclooxygenase (indomethacin) [55], and phospholipase A2 (AACOCF3) [56]. We found no significant effect for indomethacin and AACOCF3. However, we found that DPI significantly inhibited the effect of DDC, suggesting that the NADPH oxidase system may also participate in the accumulation of the superoxide anion that may be involved in the proapoptotic effect of DDC. Finally, we also found that catalase and deferoxamine [57] significantly inhibited the effect of DDC, suggesting a role of hydrogen peroxide and the hydroxyl

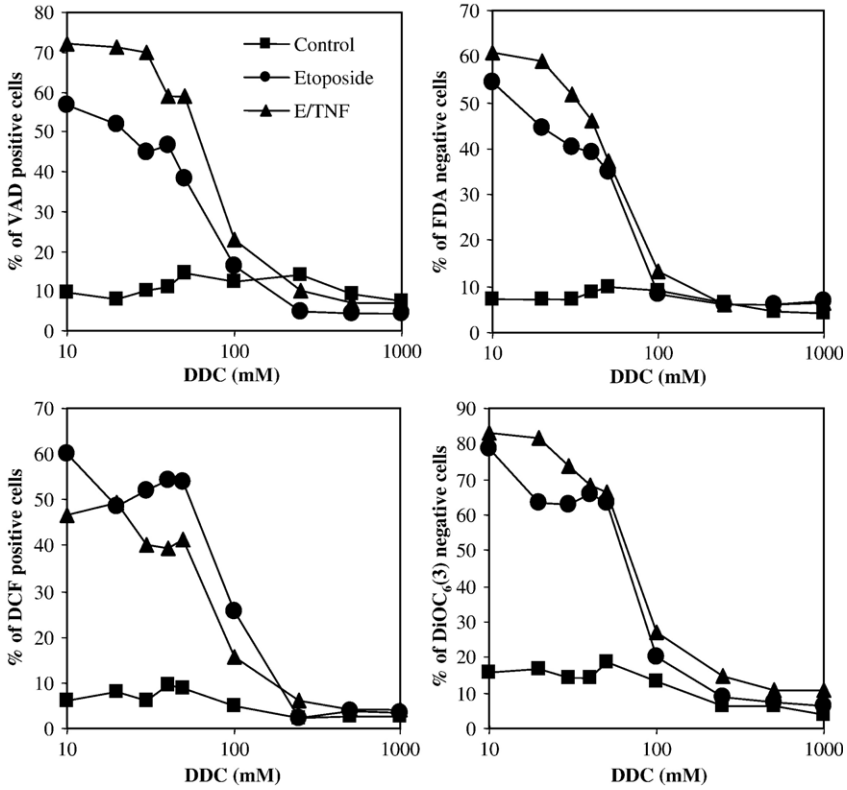

Fig. 4. Influence of DDC concentration on the inhibition of etoposide and E/TNF-induced apoptosis. Different concentrations of DDC from 10 μM to 1 mM were incubated for 16 h with HeLa control cells or HeLa cells induced to die by the addition of etoposide or E/TNF. The effect of DDC on cell death was measured in terms of caspase activation (CaspaTag, VAD-positive cells), metabolism arrest (FDA-negative cells), high ROS production (DCF-positive cells), and loss of $\Delta\Psi m$ ($DiOC_6(3)$-negative cells).

radical. These results clearly show that the DDC-induced Bax and cyt *c* translocations are ROS dependent.

We also examined whether the antiapoptotic effect of DDC was also ROS dependent. We tested the effects of the same drugs as before but on the DDC-dependent inhibition of apoptosis, induced by E/TNF and etoposide. We found that inhibitors of the respiratory chain, such has rotenone, or antioxidants such as tiron, DPI, indomethacin, AACOCF3, catalase, and DFO, had no influence on the DDC-dependent inhibition of etoposide or E/TNF-induced apoptosis (data not shown). These results suggested that, unlike its proapoptotic function, the antiapoptotic function of DDC is not primarily ROS dependent.

Since DDC acts as a copper chelator to inhibit the Cu, Zn SOD, we have also tested the role of copper (Cu) on the pro- and antiapoptotic functions of DDC. Magnesium (Mg) served as a negative control. In order to prevent the inhibition of Cu, Zn SOD by DDC, we have first used equimolar doses of DDC and Cu. However, DDC and Cu both at 500 μM led to the formation of a brown precipitate (which was not obtained with Mg) that triggers rapid necrosis (data not shown). Thus we used Cu (and Mg) at 50 μM, a concentration that did not provoke cellular toxicity. The overall SOD activity was measured in HeLa cells incubated in the presence or in the absence of DDC, coincubated or not with Cu or Mg. The results presented in Fig. 5A show that 500 μM DDC efficiently inhibits SOD activity (more than 80%), suggesting that the major SOD activity detected in HeLa cells is dependent on Cu, Zn SOD. Interestingly, the addition of Cu strongly inhibits the effect of DDC whereas Mg has no effect. Next, we have determined the effect of Cu and Mg on DDC-induced translocations of Bax and cyt *c*. Fig. 5B shows that Cu significantly inhibits the translocations of Bax and cyt *c* induced by DDC, indicating that the proapoptotic function of DDC actually depends on the inhibition of Cu, Zn SOD. It seems that Mg has also a small but significant effect ($P = 0.0395$) but we still ignore the origin of this effect. Finally, Fig. 5C shows that Cu fails to inhibit the antiapoptotic function of DDC, suggesting that this function is independent of the Cu, Zn SOD activity. These results show that, unlike its proapoptotic function, the antiapoptotic function of DDC would not depend on the inhibition of Cu, Zn SOD.

The antiapoptotic function of DDC affects both caspase activation and activity through a mechanism involving the oxidation of thiol groups

Our previous results suggest that the antiapoptotic function of DDC occurs downstream from the cyt *c* release because we have observed living cells with unfragmented nuclei and translocated Bax and cyt *c*. This in turn suggests that DDC can inhibit caspase activity and/or activation. Indeed, caspases are required for apoptosis and several nucleases, such as DFF (DNA Fragmentation Factor) or CAD (Caspase-Activated

Table 1
Effects of different antioxidant and prooxidant molecules, ROS scavengers, and inhibitors of ROS production on DDC-dependent Bax and cyt *c* translocations

Drug added to DDC	Known function	Concentration used	% of cells with translocated Bax/cyt c	*P* values
DDC (alone)	Cu, Zn SOD inhibitor	0.5 mM	28.6 ± 5.3	
Tiron	Superoxide scavenger	1 mM	0	0.0008
Rotenone	Respiratory chain complex I inhibitor	0.1 μM	3.9 ± 1.7	0.0016
NaN_3	Respiratory chain complex IV inhibitor	0.5 mM	39.5 ± 4.4	0.052
DPI	NADPH oxydase inhibitor	2.5 μM	6.2 ± 2.6	0.003
Indomethacin	Cyclooxygenase inhibitor	10 μM	25.8 ± 1.2	0.425
AACOCF3	Phospholipase A2 inhibitor	10 μM	29.3 ± 4.3	0.855
Catalase	Catalyses: $H_2O_2 \rightarrow H_2O + 1/2\ O_2$	1000 U/ml	14.8 ± 1.4	0.012
DFO	Fenton reaction inhibitor	10 μM	14.3 ± 4.3	0.023

DDC alone or with drugs affecting ROS accumulation into the cells was incubated with HeLa cells. The percentage of cells with translocated Bax and cyt *c* proteins was determined by immunofluorescence, 16 h after the start of treatment. Known functions and concentrations of the drugs are indicated. The average values and standard deviations were calculated from three independent experiments. *P* values were calculated between cells treated with the different drugs and cells treated with DDC alone. $P < 0.05$ was accepted as significant.

DNAse), are known to participate in apoptosis, and are directly or indirectly activated by caspases.

DDC is a thiol-containing molecule and could therefore inactivate caspases by interacting with their catalytic sites. It has been shown that disulfiram, another dithiocarbamate, can directly inhibit caspase-3 by forming disulfides links with it [34,35]. Therefore, we used DTT as a thiol reducing agent and found that it inhibited the antiapoptotic effect of DDC (Fig. 6). As all of the previously used antioxidants did not have any effect, this does not seem to be linked to a secondary antioxidant activity of DTT.

As E/TNF and etoposide-induced apoptosis both involve the mitochondria, and the activation of caspase-9, we attempted to characterize the effect of DDC on caspase-9 activation. The results of Western blotting with antibodies against several processed forms of this caspase showed that control HeLa cells, which were not incubated with E/TNF or etoposide, only exhibited the proform of caspase-9 (45 kDa) (Fig. 7). This proform was not modified by the addition of DDC or DTT alone. When E/TNF or etoposide was added, two intermediate processed forms (37 and 35 kDa) as well as a fully processed form (17 kDa) of caspase-9 were present in the cellular extracts. This suggests that caspase-9 was activated in both types of apoptosis. The addition of DDC strongly decreased the amount of the two intermediate processed forms of caspase-9, and converted the active 17 kDa form into another 19 kDa form. The effect of DDC on the proform was different between cells incubated with E/TNF and with etoposide. Indeed, DDC decreased the level of the proform in cells incubated with E/TNF but did not in the presence of etoposide. Nevertheless, these results clearly show that DDC alters the processing of caspase-9 in both cases, leading to the production of an abnormal form of caspase-9. Our results also show that DTT reverses this effect.

We measured the influence of DDC on effector caspases by Western blotting with an antibody raised against the poly(ADP-ribose) polymerase (PARP) protein (Fig. 7). PARP is a well-known target of effector caspases such as caspase-3 [58,59]. In control HeLa cells, we observed only the uncleaved 116-kDa PARP protein, and DDC or DTT alone had no effect on it. When E/TNF or etoposide was added, we observed a cleaved form (85 kDa), showing that effector caspases are activated under both conditions. The addition of DDC strongly decreased the amount of the cleaved form, showing that DDC directly or indirectly inhibited effector caspases. The addition of DTT partially restored the activity of the effector caspases that cleave PARP. These results suggest that DDC could exert its antiapoptotic activity through the formation of disulfides with caspases, which inhibits both caspase activation and activity.

Discussion

Here, we have shown that the inhibitor of Cu, Zn SOD, diethyldithiocarbamate, has two antagonistic and dissociable effects on apoptosis regulation. First, we have observed that DDC exerts a strong antiapoptotic effect on the extrinsic and intrinsic pathways of apoptosis, induced by E/TNF and etoposide. Second, we have shown that DDC triggers the translocation to the mitochondria of the proapoptotic Bax protein and the release of cyt *c* into the cytosol, in the absence of any other inducer of apoptosis. This property of DDC does not lead directly to cell death as DDC exerts its antiapoptotic activity at the same time, downstream from the cyt *c* release. However, we have found that the DDC-induced Bax and cyt *c* translocation is proapoptotic, which is apparent when the antiapoptotic function of DDC is inhibited, showing that DDC has the ability to trigger the intrinsic and mitochondrial-dependent pathway of apoptosis.

The antiapoptotic function of DDC appears to be ROS independent, as its effect was not modulated by any of the antioxidants used in this study. This is consistent with the observation that copper, which reactivates Cu, Zn SOD in the presence of DDC, does not modify the antiapoptotic function of DDC. We observed that DDC inhibited the activation of initiator caspases, such as caspase-9, and the activity of effector caspases, such as caspase-3. This can be reversed by DTT. Thus, this process probably involves the formation of disulfide links between DDC and caspases, which is similar to that for disulfiram [34,35]. However, several mechanisms may be involved in the DDC-dependent inhibition of caspases. First, DDC may directly react with the thiol group of caspase catalytic sites. Second, we showed that in the presence of etoposide or E/TNF, treatment with DDC results in a new processed 19-kDa form of caspase-9. It is possible that this form is produced after an inappropriate cleavage of the proform and inhibits the formation of the fully active caspase-9. Finally, we observed

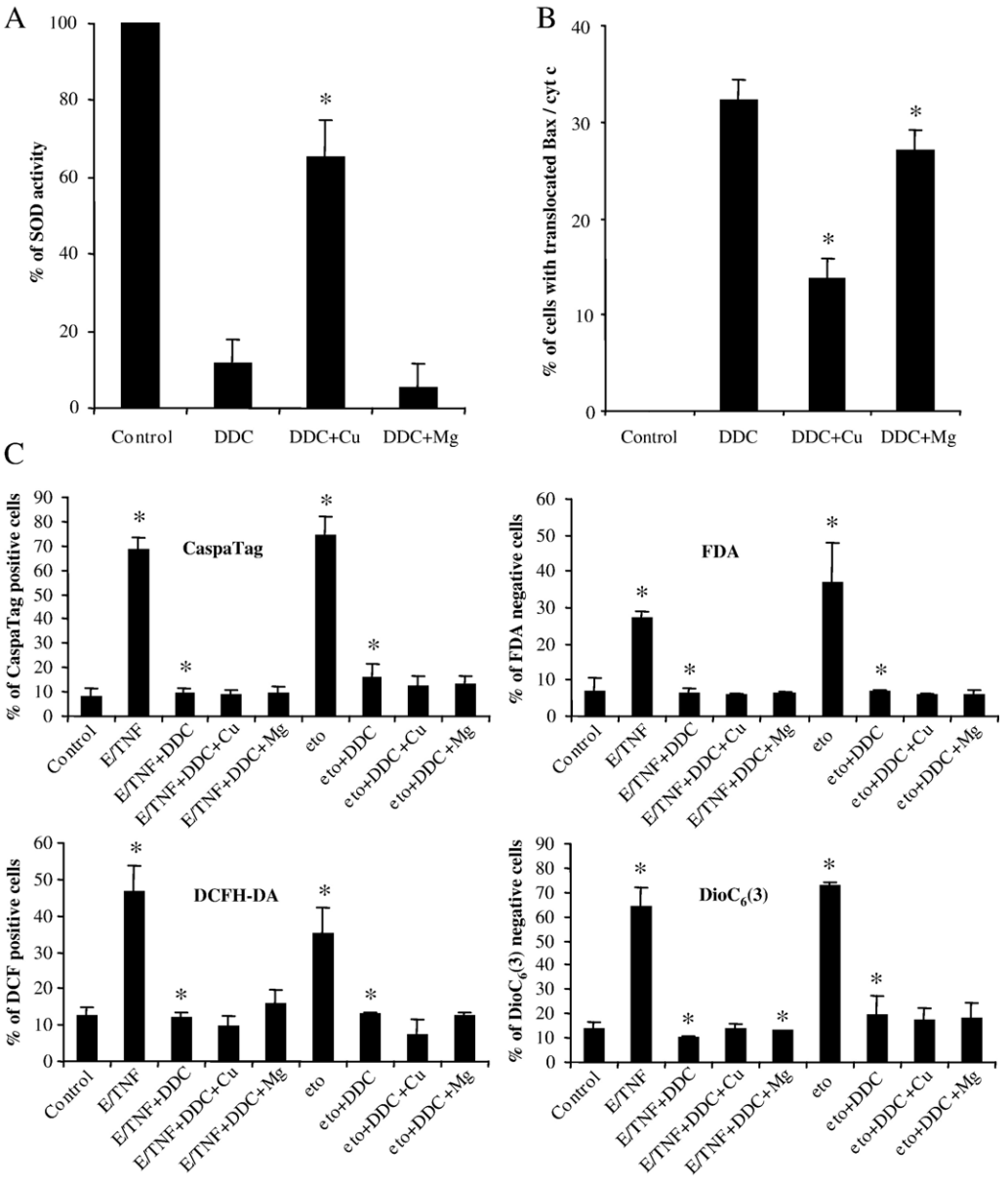

Fig. 5. Effects of copper (Cu) and magnesium (Mg) on SOD activity, DDC-induced Bax, and cyt *c* translocations and DDC-dependent inhibition of apoptosis. The SOD activity was measured in HeLa control cells or HeLa cells incubated with 500 μM DDC, in the presence or in the absence of Cu ($CuSO_4$ 50 μM) or Mg ($MgSO_4$ 50 μM). The corresponding values of SOD activity, measured 16 h after the beginning of the treatment, are presented in panel A. The percentage of cells with translocated Bax and cyt *c* proteins was determined under the same conditions and are presented in panel B. Next, Cu or Mg was added to HeLa cells at the same time and then E/TNF or etoposide +DDC. The effects of Cu and Mg were measured 16 h after the beginning of the treatment, in terms of caspase activation (CaspaTag, VAD-positive cells), metabolism arrest (FDA-negative cells), high ROS production (DCF-positive cells), and loss of $\Delta\Psi m$ ($DiOC_6(3)$) (C). The average values and standard deviations were calculated from three independent experiments. *Differences statistically significant ($P < 0.05$) compared to appropriate controls (E/TNF or etoposide versus control, E/TNF or etoposide +DDC versus E/TNF or etoposide, and E/TNF or etoposide +DDC+Cu or Mg versus E/TNF or etoposide +DDC).

that after treatment with DDC, the amount of the proform of caspase-9 decreases in the presence E/TNF, whereas it remains stable with etoposide. This suggests that, in the presence of E/TNF, caspase-9 degradation may be occurring.

The proapoptotic function of DDC is ROS dependent, as several pro- or antioxidant molecules modulated its activity. Tiron, catalase, and DFO, which reduce the levels of superoxide anion, hydrogen peroxide, and hydroxyl radicals, respectively, inhibit Bax and cyt *c* translocations induced by DDC. This suggests that different types of ROS mediate the proapoptotic function of DDC. However, superoxide anion may be central to this process because tiron completely suppresses the effect of DDC, whereas catalase and DFO only partially inhibits its effect. Furthermore, we have shown that the proapoptotic function of DDC is dependent on its ability to inhibit Cu, Zn SOD since the addition of copper, which reactivates Cu, Zn SOD, also inhibits Bax and cyt *c* translocations.

The apoptosis triggered by E/TNF or etoposide modifies the physiology of the cell in several ways. These include an increase in the production of ROS, translocations of Bax and cyt

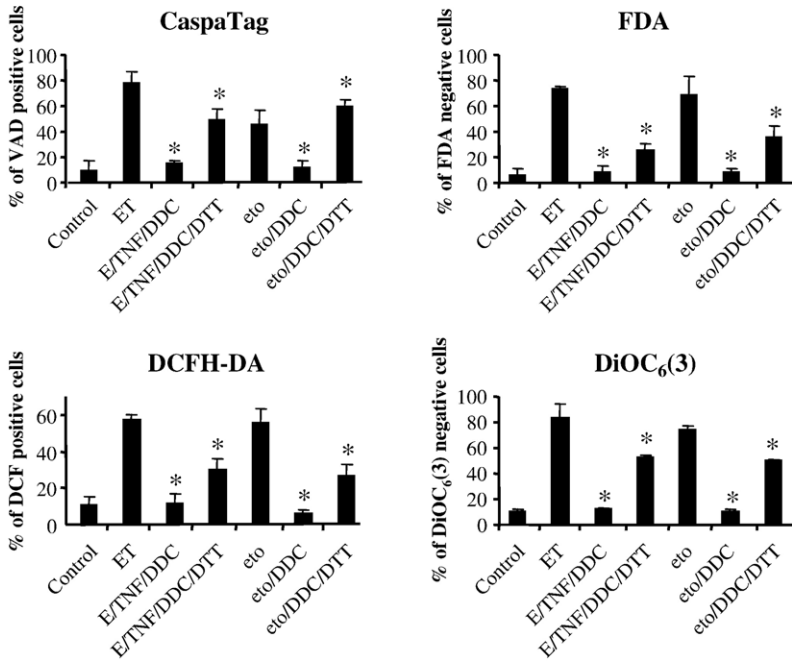

Fig. 6. Effects of DTT on DDC-dependent inhibition of apoptosis. DTT was added to HeLa cells protected from E/TNF or etoposide-induced apoptosis by the presence of DDC. The effect of this drug was measured in terms of caspase activation (CaspaTag, VAD-positive cells), metabolism arrest (FDA-negative cells), high ROS production (DCF-positive cells), and loss of $\Delta\Psi$m ($DiOC_6(3)$). The average values and standard deviations were calculated from three independent experiments. *$P < 0.05$ for E/TNF/DDC versus E/TNF, etoposide/DDC versus etoposide, E/TNF/DDC/DDT versus E/TNF/DDC, and etoposide/DDC/DDT versus etoposide/DDC, according to Student's *t* test.

c, activation of caspases, loss of $\Delta\Psi$m, nuclei fragmentation, and loss of metabolic activity. The inhibition of caspases by zVAD inhibits all these aspects of apoptosis, except for etoposide, where zVAD does not inhibit the translocations of Bax and cyt *c*. This observation could be related to the fact that activation of caspases is the first step in the extrinsic pathway of apoptosis, whereas it only occurs after the release of cyt *c* into the cytosol in the intrinsic pathway. DDC has the same effect as zVAD (except for the Bax and cyt *c* translocations in E/TNF-induced apoptosis). This reinforces the idea that the antiapoptotic activity of DDC is due to its ability to inhibit caspases. However, they also show that ROS production, measured with DCFH-DA, and loss of $\Delta\Psi$m and cell viability are caspase dependent.

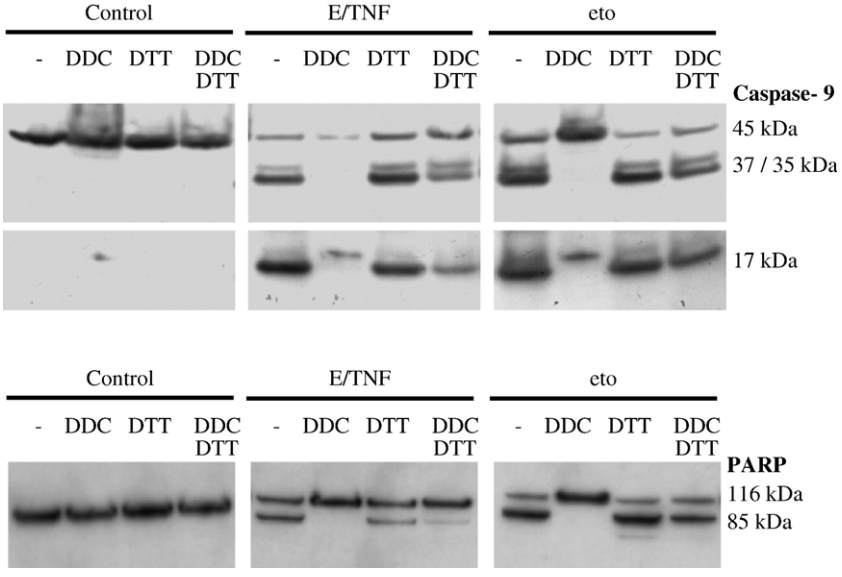

Fig. 7. Effects of DDC and DTT on caspase-9 activation and PARP cleavage. Western blotting was carried out with anti-caspase-9 and anti-PARP antibodies, on proteins obtained from cellular extracts of HeLa cells treated or not with E/TNF or etoposide, and with or without DDC and DTT. With the anti-caspase-9 antibodies, four forms of caspase-9 can be distinguished: the 45-kDa proform, two intermediate 37- and 35-kDa processed forms, and a 17-kDa full processed form. With the anti-PARP antibodies, two forms of PARP can be distinguished: the full-length 116-kDa PARP and its caspase-cleaved 85-kDa form.

If ROS production were inhibited by zVAD for both types of apoptosis, the production of ROS would occur after the activation of caspases. However, the fact that the antioxidant BHA inhibits most aspects of apoptosis demonstrates that ROS are important accelerators of the apoptotic process. In both types of apoptosis, the activation of caspases would trigger a large production of ROS, accelerating Bax and cyt *c* translocations and caspase activation, as well as the loss of metabolic activity. We have previously shown with HeLa cells having (ρ^+) or lacking (ρ^0) a functional mitochondrial respiratory chain that BHA preferentially inhibits the mitochondrial-dependent production of ROS [13]. As BHA inhibits ROS production and apoptosis in this system, and as HeLa ρ^0 cells are less sensitive to E/TNF and etoposide ([13] and unpublished data), ROS production may originate from the mitochondrial respiratory chain.

There have been several studies on the regulation of apoptosis by dithiocarbamates. About half of these studies describe an inhibitory effect of dithiocarbamates on apoptosis, whereas the other studies show that dithiocarbamates exhibit proapoptotic properties. It was shown that pyrrolidinedithiocarbamate (PDTC), DDC, and dimethyldithiocarbamate (DMDTC) inhibit UV-induced apoptosis in HL60 cells [60] and that disulfiram inhibits TNF-α-induced apoptosis in LLC-PK1 cells [61], etoposide-induced apoptosis in rat thymocytes [34], and Fas-induced apoptosis in Jurkatt cells [35]. However, some studies have shown that PDTC induces a cyt *c*-dependent apoptosis in HL60 cells [62], that disulfiram induces apoptosis in human melanoma cells [63], that DDC induces apoptosis in leukemia cell lines [64], in rat thymocytes [65], or in vascular smooth muscle cells [32], and both apoptosis and necrosis in HL60 cells [66]. The effect seen does not seem to depend on the concentration of dithiocarbamate as both effects have been obtained with both low and high concentrations of dithiocarbamates. We have shown that DDC can have simultaneous pro- or antiapoptotic activities. Thus, it is possible that either property of DDC could be inhibited or favored depending on the cell type or culture conditions used. For example, low activities of superoxide production systems, such as the mitochondrial respiratory chain or NADPH oxidase, would favor the antiapoptotic effect of DDC, whereas redox regulation systems such as thioredoxin [67], in a high reducing state, would favor the proapoptotic effect of DDC. When both properties function, the antiapoptotic effect would be dominant, and cyt *c* would be released into the cytosol but the cells would remain alive. In this study, we observed that HeLa cells can be maintained for several days in culture with free cyt *c*, without showing any sign of apoptosis (data not shown). In this way, it has been suggested that the release of cyt *c* from the mitochondria can occur in a two-step process [41]. In the first step, only a small amount of cyt *c* is released, allowing the mitochondrial respiratory chain to function whereas in the second step, the release of cyt *c* would be accelerated by caspase activation and ROS production, also leading to $\Delta\Psi$m loss.

In our study, several results suggest that Bax translocation to the mitochondria, which is known to participate in releasing cyt *c* from the intermembrane space, is ROS dependent. The inhibition by BHA of Bax translocation suggests that mitochondrial ROS can trigger Bax translocation and the ROS-dependent triggering of Bax translocation by DDC is completely inhibited by tiron, a superoxide scavenger. Unfortunately, the mechanism of cyt *c* release from the mitochondria remains partly unexplained. Several models propose that proapoptotic proteins of the Bcl-2 family homo- or hetero-oligomerize, whereas others propose that these proteins could interact with other mitochondrial proteins, to form pores that allow the release of the cyt *c* from the mitochondria [68–70]. Studies have shown that this pore formation is not sufficient and that the oxidation of cardiolipins, where cyt *c* is anchored, is also necessary [41,71]. The posttranslational modifications allowing Bax translocation to mitochondria are still unclear. Many studies have suggested that its interaction with other proapoptotic homologs is crucial [72,73] and others have suggested that the phosphorylation state of Bax is also important [74]. Our study suggests that ROS, and perhaps superoxide anion, may directly or indirectly participate in the mechanism of Bax translocation. Most studies on the relationship between Bax and ROS show that Bax translocation can trigger the production of ROS [15,75], whereas very few studies have looked at how ROS could translocate Bax to the mitochondria [76]. Therefore, the DDC-induced translocation of Bax in HeLa cells may prove important for studying the effects of oxidants, such as the superoxide anion, on the mediators of apoptosis, such as Bax and related proteins. The oxidation of particular domains in these proteins may be involved, favoring a conformational change of Bax and its translocation to the mitochondria. Finally, it is often unclear whether ROS involvement during apoptosis is a primary effect in the apoptotic machinery or a secondary mechanism, in response to caspase activation. As DDC also inhibits caspase activity in this system, we can use this advantage to study the primary effects of ROS in the translocation of Bax to the mitochondria.

Acknowledgments

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