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Mitochondrial-Targeted Fatty Acid Analog Induces Apoptosis with Selective Loss of Mitochondrial Glutathione in Promyelocytic Leukemia Cells

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Summary

Some fatty acids and derivatives are known to induce cell death in cancer cells. Mitochondria may have important roles in the death process. Therefore, we investigated the mitochondrial contribution in cell death induced by a modified fatty acid, tetradecylthioacetic acid (TTA), which cannot be β-oxidized. TTA treatment induced apoptosis in IPC-81 leukemia cells via depolarization of the mitochondrial membrane potential ($\Delta\psi$) and early release of cytochrome c, accompanied by depletion of mitochondrial glutathione. Caspase-3 activation and cleavage of poly (ADP-ribose) polymerase (PARP) occurred at a late stage, but the broad-spectra caspase inhibitor zVAD-fmk did not block TTA-induced apoptosis. Overexpression of Bcl-2 partially prevented TTA-induced apoptosis, whereas cAMP-induced cell death was completely blocked. In conclusion, TTA seems to trigger apoptosis through mitochondrial-mediated mechanisms and selective modulation of the mitochondrial redox equilibrium.

Introduction

Apoptotic stimuli trigger coordinated action of specific cascades involving different organelles and structures, such as the nucleus, the endoplasmic reticulum, the mitochondria, and the plasma membrane. The understanding that mitochondria are key organelles in apoptosis induced by various stimuli has evolved from observations of changes in mitochondrial membrane integrity and release of mitochondrial substances, which seem to be rate limiting for induction of caspases and endonucleases [1]. Mitochondrial membrane permeabilization is accompanied with depletion of the membrane potential ($\Delta\psi$) and release of matrix solutes and intermembrane proteins, including the apoptosome-activating cytochrome c [2] and Diablo/SMAC-1 [3, 4]. Members of the Bcl-2 family proteins regulate the apoptotic pathway involving the mitochondria through protein-protein interactions [5] with proteins such as the adenine nucleotide translocator (ANT) and the voltage-dependent anion channel (VDAC) controlling mitochondrial permeabilization [1, 6–9]. In addition, the Bcl-2 protein has been suggested to function in an antioxidant pathway to prevent apoptosis [10].

Glutathione is of vital importance for maintenance of cellular redox homeostasis, and deprivation of glutathione typically results in severe oxidative damage associated with mitochondrial degeneration (reviewed in [11]). Activation of apoptosis may occasionally include increased generation of reactive oxygen species (ROS) [12], and is sometimes accompanied by cellular glutathione depletion [13–15]. In accordance, experimental modulation of cellular thiols has been reported to affect the cellular resistance to apoptosis [16, 17], supporting the theory that increased levels of glutathione may be associated with drug and radiation resistance of malignant cells [11, 18].

Mitochondria do not have the ability to produce glutathione, which is required for the normal function of these organelles [11]. The transfer of glutathione from cytosol into mitochondria is mediated by a multicomponent transport system. This system provides a remarkable ability of mitochondria to retain glutathione under conditions where the cytosolic level might fall [19]. The mitochondrial thiol status has been proposed to constitute a critical sensor of the cellular redox potential that is of importance during mitochondrial membrane permeabilization and induction of apoptosis [16, 20–22].

The promyelocytic leukaemia cell line IPC-81 undergoes rapid and synchronous apoptosis when treated with cAMP-elevating agents or cell-permeable cAMP analogs [23]. The cAMP-induced apoptosis occurs through activation of cAMP-dependent kinase, involving phosphorylation of transcription regulators, protein synthesis and cotranslational phosphorylation [24–26]. IPC-81 cells overexpressing Bcl-2 (IPC-81 Bcl-2) or inducible cAMP early repressor (ICER; IPC-81 ICER) are protected against cAMP-induced apoptosis, but IPC-81 Bcl-2 cells undergo granulocytic differentiation during long-term exposure to cAMP [25, 27].

A range of compounds involved in cellular lipid metabolism, including saturated and unsaturated fatty acids and derivatives, induce apoptosis [28–32]. It has been suggested that cell death induced by long-chain fatty acids in pancreatic islets is conducted via a lipoapototic pathway involving ceramide synthesis and NO overproduction [33]. In another system, palmitic acid was found to induce apoptosis via a direct effect on mitochondria [32]. Fatty acids may cause changes in mitochondrial membrane permeability due to their protonophoric activity and/or through their interaction with ANT [34–36] and other ion transport systems of the inner membrane.

TTA is a modified saturated fatty acid analog with a sulfur atom inserted in the 3 position from the carboxylic end (CH_3-$(CH_2)_{13}$-S-CH_2COOH). The metabolism and metabolic effects of TTA have been reviewed [37, 38]. This thia-fatty acid is blocked for β-oxidation and is

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therefore catabolized by the ω-oxidation pathway in endoplasmic reticulum and peroxisomes. As a ligand for members of the peroxisome proliferator-activated receptor (PPAR) family of nuclear hormone receptors, TTA regulates expression of fatty acid-metabolizing enzymes, in particular those of catabolic pathways [37, 39, 40]. Following cellular uptake, TTA is converted to TTA-CoA [41], which is a substrate for the carnitine palmitoyltransferase system (CPT I and CPT II) transporting acyl-CoAs into the mitochondria [37, 42]. TTA stimulates the mitochondrial oxidation of normal fatty acids [43] and induces mitochondrial proliferation in rat hepatocytes [44].

Antiproliferative effects of TTA have been observed in several cancer cell lines, and circumstantial evidence for induction of apoptosis has been found [45–48]. In this study, we compare apoptosis induced by cAMP and TTA in a myeloid leukemia cell line. Since TTA-induced apoptosis was partly blocked by Bcl-2 overexpression and involved early mitochondrial changes, we suggest that mitochondria participate in the death process.

Results

Induction of Apoptosis after Treatment with TTA

Apoptotic cell surface blebbing was observed in parallel with chromatin condensation and nuclear fragmentation in IPC-81 cultures exposed to TTA. Morphological signs of TTA-induced apoptosis were evident after 12 hr (18.6% ± 10.1%), and the percentage of apoptotic cells gradually increased until it reached a plateau around 20 hr (67.2% ± 4.2%) after TTA-addition (Figure 1A). Cells overexpressing Bcl-2 were protected from apoptosis at early stages, but a moderate increase in the apoptotic score was observed at later stages (20 and 24 hr). Apoptotic induction by the cell permeable cAMP analog 8-chlorophenylthio-cAMP (8CPT-cAMP) in IPC-81 cells was more rapid than with TTA, but it was completely blocked by overexpression of Bcl-2 (Figure 1B).

TTA Triggers Cytochrome c Release

It is widely accepted that cytochrome c release is a key event in mitochondrial-mediated apoptosis, leading to activation of executive cascades. In our studies, the level of cytochrome c in mitochondrial fraction of IPC-81 cells fell nearly 70% within 6 hr of TTA exposure, after which it remained at a low level (Figure 2A). The level of cytochrome c in the cytosolic fraction increased in conformity with the mitochondrial loss (Figure 2B). The inserted curve in Figure 2B indicates that most of the cytochrome c was released during the first 3 hr of treatment. In comparison, the mitochondrial level of cytochrome c in 8CPT-cAMP-treated cells gradually declined to about 30% after 6 hr of incubation, simultaneously with an increasing level in the cytosol (Figures 2C and 2D). Overexpression of Bcl-2 or ICER did not block TTA-induced translocation of cytochrome c, whereas the effect of 8CPT-cAMP was absent in these cells (Figures 2E and 2F).

The Role of Caspases in TTA-Induced Apoptosis

Release of cytochrome c to the cytosol leads to activation of caspase-3 and, thereby, proteolysis of distinct

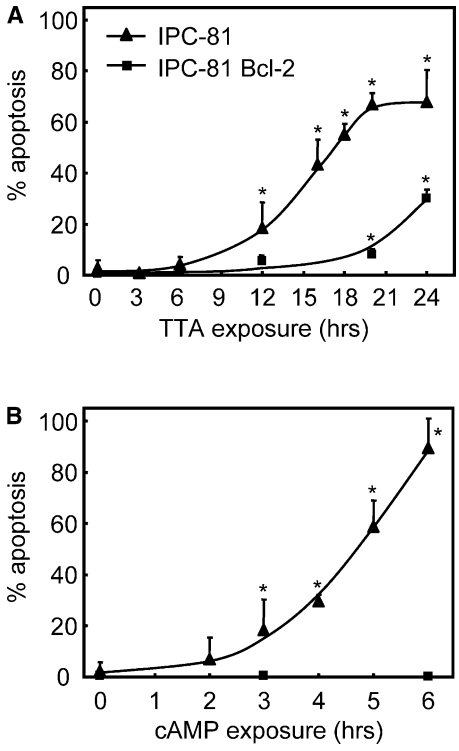

Figure 1. Induction of Apoptosis in Cells Treated with TTA and 8CPT-cAMP

Wild-type and Bcl-2-overexpressing IPC-81 cells were treated with either 500 μM BSA-bound TTA (A) or 200 μM 8CPT-cAMP (cAMP) (B). Apoptosis was determined by microscopic examination of cell surface and nuclei after staining with Hoechst 33342. The data are presented as mean ± SD. *$p < 0.05$ compared to untreated control.

substrates. TTA induced the activity of caspase-3 in IPC-81 cells (Figure 3A), and this occurred at late stages about simultaneously with the appearance of apoptotic morphology (Figure 1A). A similar pattern was also observed for 8CPT-cAMP (Figures 3A and 1B). Activation of caspase-3 with TTA was supported by a decrease in the level of the inactive procaspase-3 and cleavage of the caspase-3 substrate protein poly(ADP-ribose) polymerase (PARP), which is a nuclear enzyme that detects and binds DNA strand breaks produced by various apoptotic stimuli (Figure 3B). A marked decrease in the procaspase-3 level and appearance of the apoptotic 85 kDa PARP cleavage product occurred between 12 and 18 hr of TTA treatment, simultaneously with the dramatic increase in caspase-3 enzyme activity (Figure 3A). In 8CPT-cAMP-treated cells, the level of procaspase-3 declined simultaneously with elevated caspase-3 activity and cleavage of PARP (Figure 3B). To decide whether caspase activation is essential in the apoptotic execution, we examined the influence of the broad-spectra caspase antagonist zVAD-fmk. After 20 hr of TTA exposure, the percentage of cells with apoptotic morphology did not seem to be affected by the presence of zVAD-fmk (Figure 3C). However, a lower degree of nuclear fragmentation was observed in apoptotic cells with condensed chromatin in the presence of zVAD-fmk (Figure 3D). In contrast, zVAD-fmk efficiently counteracted 8CPT-cAMP-induced apoptosis (Figures 3C and 3D).

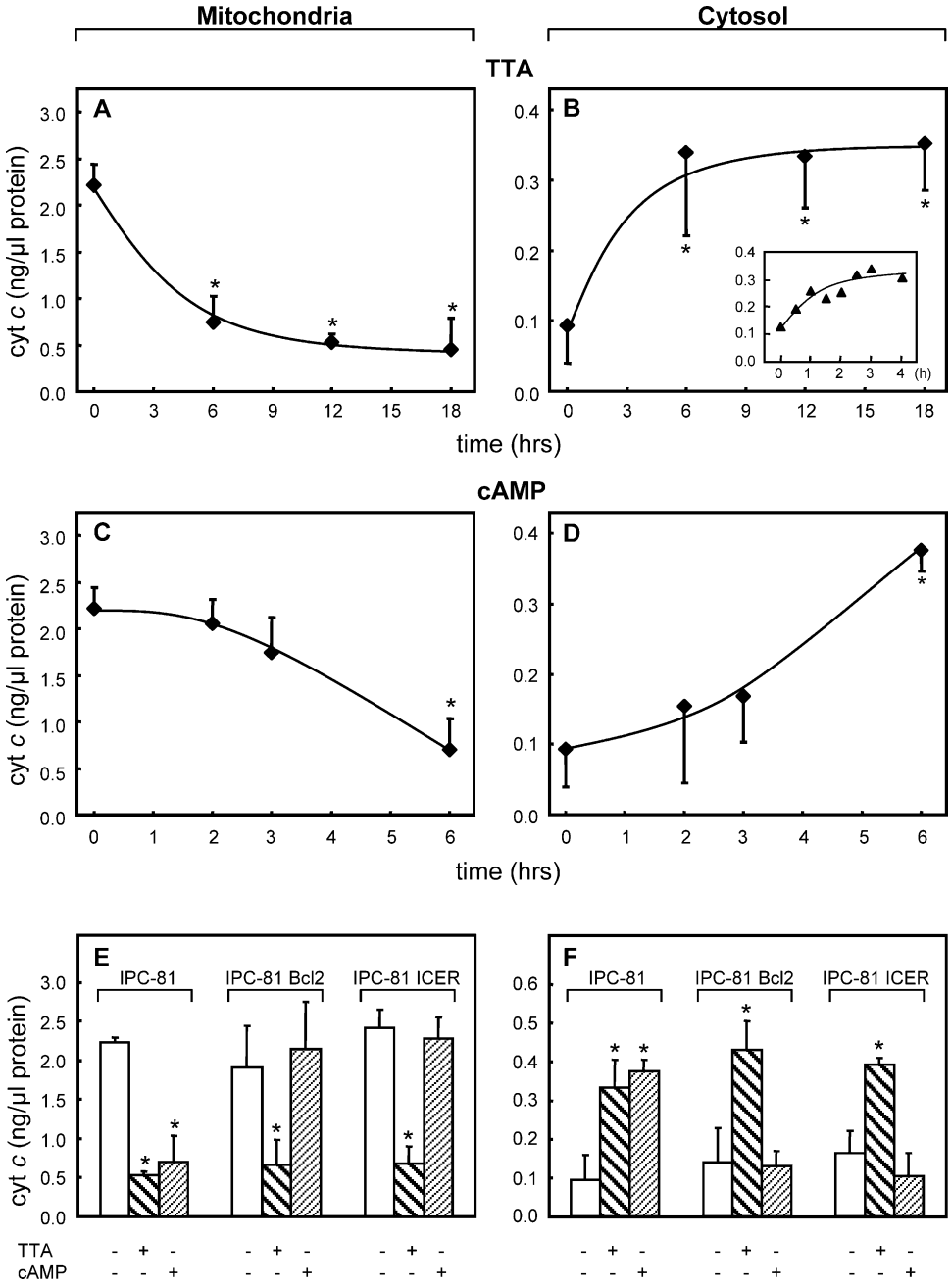

Figure 2. Cytochrome c Release

Mitochondrial and cytosolic fractions were isolated from wild-type IPC-81 (wt) cells, and IPC-81 cells overexpressing Bcl-2 or ICER. Cytochrome c (Cyt *c*) was determined as described in Experimental Procedures. Cells were treated with 500 μM BSA-bound TTA (A and B) or 200 μM 8CPT-cAMP (cAMP) (C and D). The inserted curve in (B) shows the increase of cytosolic cytochrome c at early time points (0.5–4 hr) in TTA-treated cells. (E and F) Cytochrome c was measured in cytosol and mitochondria from IPC-81 wild-type, IPC-81 Bcl-2, and IPC-81 ICER cells after 12 and 6 hr of exposure to 500 μM TTA and 200 μM 8CPT-cAMP, respectively. The data are given as mean ± SD. *$p < 0.05$ compared to untreated control.

Alterations in Mitochondrial Glutathione Status during Apoptosis

Glutathione is a key regulator of the intracellular redox status, which is an important factor in the control of cellular homeostasis. Previously, cellular glutathione depletion has been associated with apoptosis. The mitochondrial pool of glutathione is regulated separately from the cytosolic pool, and it appears to be resistant to fluctuations in the cytosolic level of glutathione [19]. We therefore investigated how the mitochondrial and cytosolic pools of glutathione were affected during TTA- and 8CPT-cAMP-induced apoptosis.

TTA caused a time-dependent decrease in the mitochondrial level of glutathione (GSH + GSSG) in IPC-

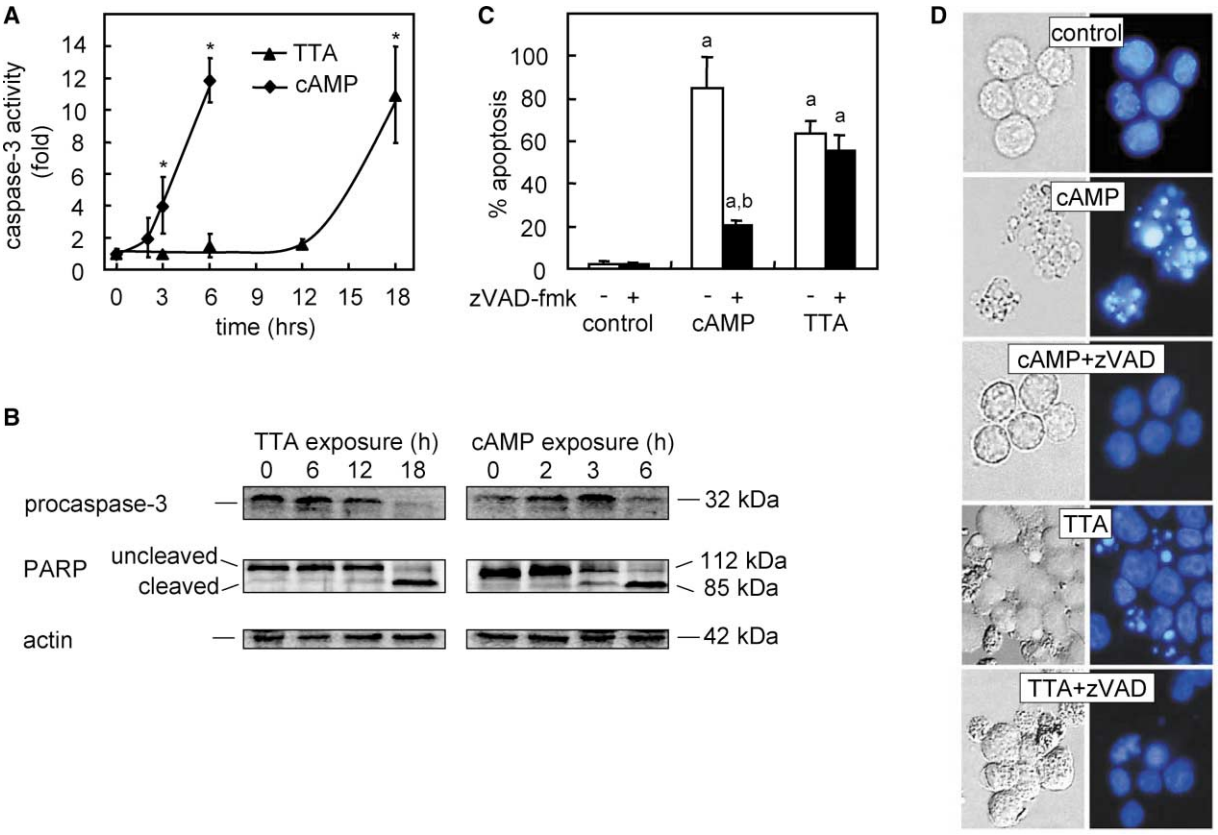

Figure 3. The Role of Caspases in 8CPT-cAMP- and TTA-Induced Cell Death

IPC-81 cells were exposed to 500 μM BSA-bound TTA or 200 μM 8CPT-cAMP (cAMP).

(A) Capase-3 activity was measured in cellular extracts.

(B) The level of procaspase-3 and cleavage pattern of PARP was assessed by Western analysis.

(C) Apoptosis was scored in cultures treated with 8CPT-cAMP (6 hr) or TTA (20 hr) in absence or presence of zVAD-fmk (zVAD).

(D) Morphological changes were visualized by phase contrast (left) and Hoechst fluorescence (right) microscopy. The data are given as mean ± SD. $^{a}p < 0.05$ compared to untreated control, $^{b}p < 0.05$ compared to the same treatment (cAMP) in absence of zVAD-fmk.

81 cells (Figure 4A). This was in part due to loss of nonoxidized GSH, leading to a significant decrease in the GSH/(GSSG + GSH) ratio at late stages (12 and 18 hr) (Figure 4B). The mitochondrial level of glutathione was kept constant during 8CPT-cAMP treatment (Figure 4C), but there was a slight decrease in GSH (significant after 6 hr) causing a moderate but significant decrease in the mitochondrial GSH/(GSSG + GSH) ratio at late stages (3 and 6 hr) (Figure 4D).

More than 90% of the free cellular glutathione was found in the cytosolic fraction, and the level of GSH + GSSG was not significantly altered during 18 hr of treatment with TTA (Figure 4E). There was, however, a late decline in GSH (Figure 4E), resulting in a moderate decrease in the cytosolic GSH/(GSSG + GSH) ratio at late stages (12 and 18 hr) (Figure 4F). Neither the level of glutathione nor the GSH/(GSSG + GSH) ratio in cytosol was affected by treatment with 8CPT-cAMP (Figures 4G and 4H).

Analysis of Morphology, $\Delta\psi$, and Glutathione Distribution by Fluorescent Probes

To obtain further evidence in support of the involvement of mitochondria and glutathione status during TTA-induced apoptosis, we examined uptake and distribution of relevant fluorescent probes by scanning laser imaging and flow cytometry. Cellular staining by some of these probes was obstructed by exogenous bovine serum albumin (BSA) used for TTA administration, possibly due to physical interaction and dye adsorption by BSA. Hence, TTA was prepared in absence exogenous BSA by binding to culture serum protein. In previous studies, the exclusion of BSA has been demonstrated to sensitize cells, possibly due to increased cellular uptake of TTA [46]. Thus, serum-bound TTA at a concentration of 200 μM induced massive nuclear fragmentation within 6 hr in IPC-81 cells (Figure 5A). The morphology of TTA-induced apoptosis was analogous to the classic apoptotic morphology observed after exposure to 8CPT-cAMP (Figure 5B), with chromatin margination and an extensive formation of micronuclei. Overexpression of Bcl-2 partly rescued the cells from TTA-mediated cell death, confirming the results in Figure 2. Apoptosis induced by 8CPT-cAMP was, in contrast, completely blocked by Bcl-2 overexpression (Figure 5B).

We examined alterations in $\Delta\psi$ by using JC-1 as described in Experimental Procedures. The red fluorescence, reflecting $\Delta\psi$-dependent JC-1 aggregation, was affected by both TTA- and 8CPT-cAMP treatment, whereas the green fluorescence remained unchanged

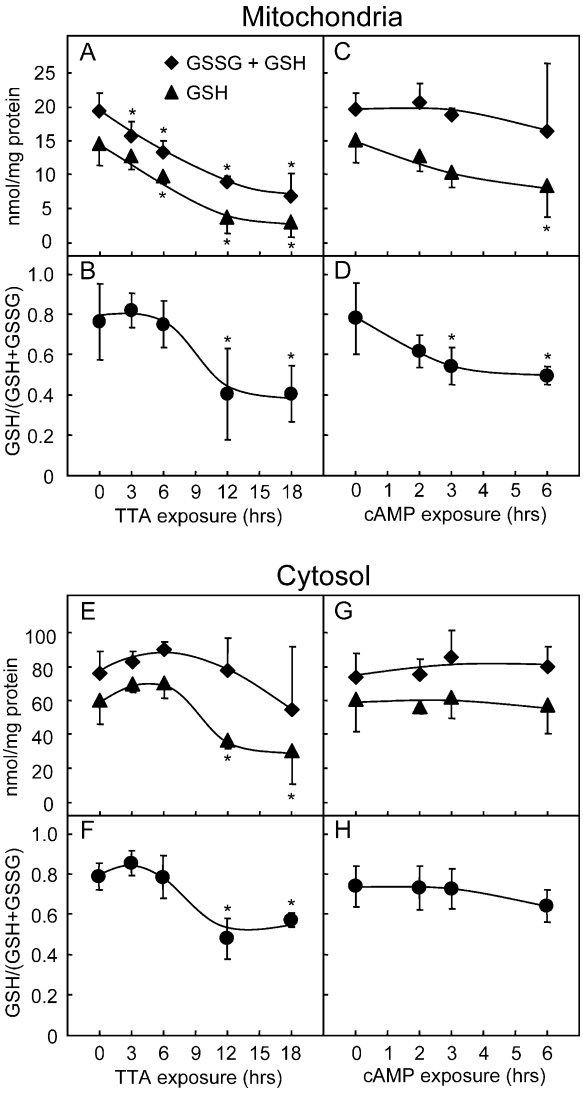

Figure 4. Modulation of Mitochondrial Glutathione Early in Apoptosis

The content of total free glutathione (GSSG + GSH) and nonoxidized free glutathione (GSH) was determined by HPLC, and the respective GSH/(GSSG + GSH) ratios were calculated in the mitochondrial (A–D) and cytosolic (E–F) fractions after treatment with 500 μM TTA (A, B, E, and F) or 200 μM 8CPT-cAMP (cAMP) (C, D, G, and H). The results are presented as mean ± SD. *$p < 0.05$ compared to untreated control.

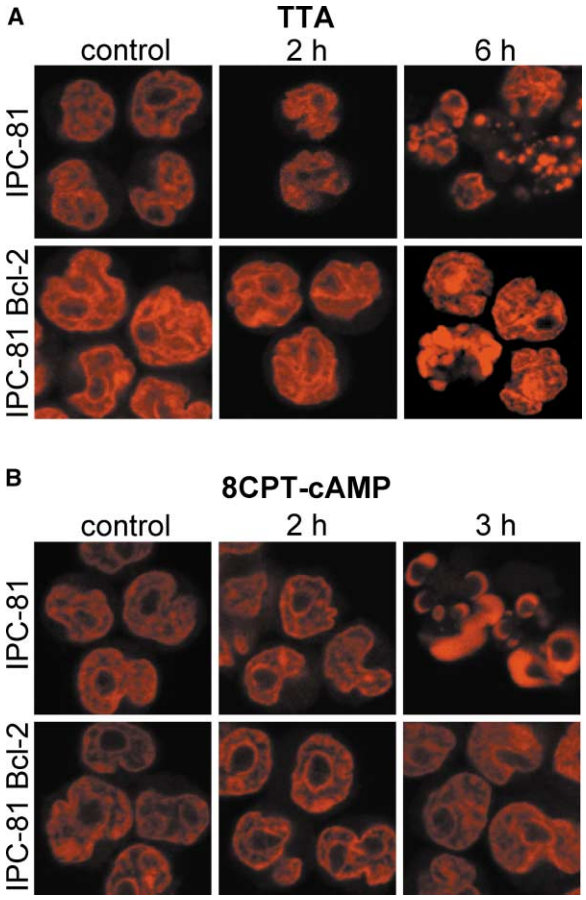

Figure 5. Apoptotic Nuclear Morphology in Wild-Type and Bcl-2-Overexpressing IPC-81 Cells

Cells were treated with 200 μM TTA bound to serum protein (A) or 200 μM 8CPT-cAMP (B). Cells were fixed in 4% paraformaldehyde with daunorubicin (5 μM), mounted on slides, and examined by laser scanning confocal microscopy

(Figure 6A). The decrease in red fluorescence demonstrates that $\Delta\psi$ was depolarized in IPC-81 cells after exposure to TTA and 8CPT-cAMP (Figure 6B). For 8CPT-cAMP-treated cells, this is consistent with previous observations that the ATP-level decreases rapidly [49]. Overexpression of Bcl-2 blocked TTA- and 8CPT-cAMP-mediated $\Delta\psi$ depletion completely, as indicated by the sustained level of red fluorescence in treated IPC-Bcl-2 cells (Figure 6C).

The above data were supported by using the $\Delta\psi$-dependent probe MTR in combination with the glutathione-reactive dye CMFDA. TTA and 8CPT-cAMP reduced the intensity of MTR in IPC-81 cells, but not in IPC-81 Bcl-2 cells (Figure 7). Furthermore, MTR staining revealed a possible relocalization of mitochondria, i.e., from an even to a clustered distribution, in IPC-81 cells exposed to 8CPT-cAMP, but not TTA. Redistribution of mitochondria in early stages of apoptosis has previously been described [50].

We observed possible subcellular alterations toward a more heterogeneous distribution of the glutathione-reactive compound CMFDA during 8CPT-cAMP, and especially, TTA treatment (Figures 7A and 7C). At late stages, there was a significant loss of cellular CMFDA intensity. The CMFDA distribution was unchanged in IPC-Bcl-2 cells exposed to 8CPT-cAMP (Figure 7D); however, the dye seemed to accumulate in specific intracellular compartments in a fraction of the TTA-treated cells (Figure 7B). These observations are in accordance with changes in the level and distribution of cellular glutathione, supporting the data in Figure 4.

Discussion

The data obtained in the present study were compatible with the hypothesis that TTA triggers apoptosis via a specific effect on mitochondria. First, the fatty acid ana-

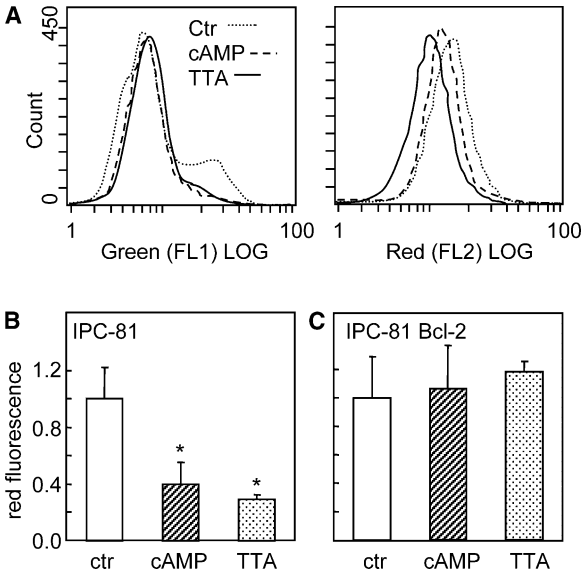

Figure 6. Depolarization of $\Delta\psi$ after Treatment with TTA and 8CPT-cAMP

Mitochondrial depolarization was determined by flow-cytometric measurements after incubation with the $\Delta\psi$-sensitive dye JC-1. Dissipation of $\Delta\psi$ is indicated by a decrease in red fluorescence (590 nm). (A) Presents representative histograms from the flow-cytometric analysis. The level of red fluorescence relative to control is presented as mean $\pm$ SD for wild-type (B) and Bcl-2-overexpressing (C) IPC-81 cells treated with either 200 μM serum protein bound TTA or 200 μM 8CPT-cAMP (cAMP). *$p < 0.05$ compared to untreated control.

log induced an early release of cytochrome c accompanied by a reduction of $\Delta\psi$. Second, TTA caused a specific time-dependent decrease in the mitochondrial level of glutathione leading to a significant decrease in the GSH/(GSSG + GSH) ratio. Caspase-3 activation and PARP cleavage were observed after cytochrome c release, and interestingly, caspase inhibition failed to reduce the effect of TTA. The only manipulation which effectively reduced TTA-induced cell death was overexpression of Bcl-2, an antiapoptotic protein whose mitochondrial effect is well documented. Moreover, it appears that Bcl-2 prevents the release of apoptogenic factors from isolated mitochondria treated with a saturated fatty acid [32]. Metabolic effects of TTA have been widely described, and mitochondria seem to be central regulators of TTA-mediated action [37, 43].

We have previously evaluated various methods for adequate fatty acid administration to cell culture [46]. By changing the method of fatty acid preparation, the cellular responses may be affected. Cells treated with TTA in the absence and presence of exogenous BSA appeared morphologically similar, but there were differences in dose response and time frame of induction. These data are in line with previous results showing that exclusion of exogenous BSA increases the cellular fatty acid uptake, allowing significant cellular responses at lower fatty acid concentrations [46].

TTA activates nuclear hormone receptors of the PPAR family [39, 51], suggesting that functional modulation may occur through transcriptional activity and protein synthesis. However, we have recently found that PPAR-independent mechanisms seem to predominate in the antiproliferative effects of TTA in glioma cells [47].

IPC-81 cells treated with TTA acquired morphological changes within the definition of apoptosis. Cytochrome c release was triggered early (Figure 2) and preceded caspase-3 activation and PARP cleavage by several hours (Figures 3A and 3B). The relatively late onset of caspase-3 activation may suggest that this occurs downstream to the commitment stage, which was supported by the minor influence of zVAD-fmk on the percentage of apoptotic cells in cultures exposed to TTA (Figure 3C). In presence of zVAD-fmk, TTA induced chromatin condensation, but apparently nuclear fragmentation was restricted (Figure 3D), proposing that zVAD-fmk-sensitive caspases may participate in the degradation phase without being crucial for the commit-

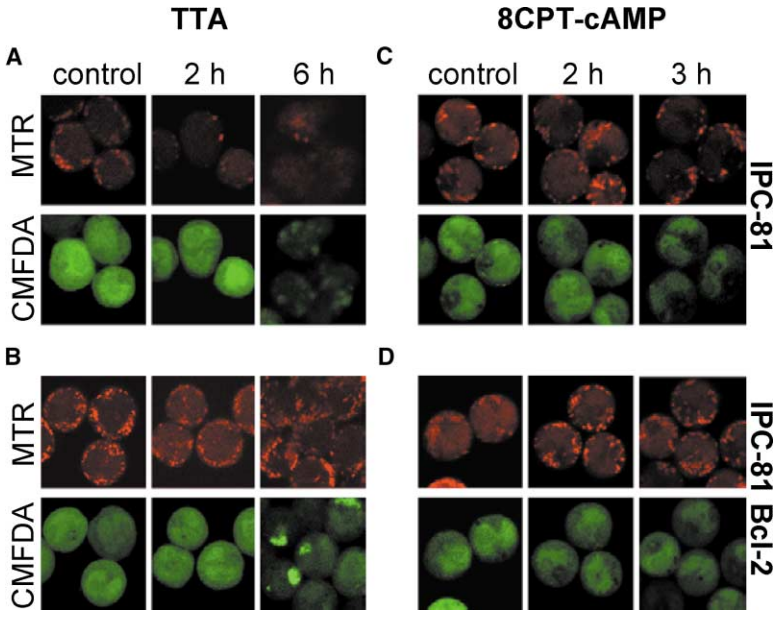

Figure 7. Mitochondrial Localization and Glutathione Distribution

Cells were labeled with MTR in combination with CMFDA as described in Experimental Procedures. The samples were studied by laser scanning confocal microscopy. Red and green fluorescence indicates mitochondria and glutathione, respectively. (A) IPC-81 wild-type, TTA treatment; (B) IPC-81 Bcl-2, TTA treatment; (C) IPC-81 wild-type, 8CPT-cAMP treatment; (D) IPC-81 Bcl-2, 8CPT-cAMP treatment.

ment. Bcl-2 overexpression significantly reduced the cellular sensitivity to TTA (Figures 1 and 5). The partly counteracting effect of Bcl-2 overexpression on TTA-induced apoptosis may be explained by subsidiary involvement of Bcl-2-dependent and Bcl-2-independent pathways. Another possibility could be that TTA-induced mechanism(s) exceeds the protective capacity of Bcl-2. It is, however, a paradox that cytochrome c release appeared equally massive in IPC-81 Bcl-2 cells compared to wild-type cells (Figure 2).

It is commonly accepted that oxidative stress may induce apoptosis, and consequently, impaired cellular antioxidant status is likely to sensitize cells to oxidative induction of apoptosis. Depletion of cellular glutathione may occur during apoptosis, but its importance in cell death remains elusive [52]. It has, however, been proposed that redox disequillibrium consequent to glutathione depletion might cause cytochrome c release [53]. These observations demonstrate that, under some circumstances, the cellular level of glutathione seems to be of importance in apoptosis. Furthermore, Bcl-2 was found to block apoptosis through nuclear glutathione sequestration [54], and manipulative depletion of mitochondrial glutathione sensitized Bcl-2-overexpressing HL-60 cells to cyanide *m*-chlorophenylhydrazone-induced apoptosis [21]. It has also been suggested that mitochondrial thiols constitute a critical cellular redox sensor in some apoptotic pathways [16]. Noteworthy, TTA induced depletion of mitochondrial glutathione early in the apoptotic process, independent of the cytosolic level of glutathione (Figure 4), which suggests that a selective modulation of the mitochondrial redox status may be of importance in TTA-induced apoptosis. Cellular imaging with CMFDA supported the occurrence of subcellular redistribution of glutathione during TTA treatment (Figure 7).

Mitochondrial permeability transition seems to be regulated by the redox status of mitochondrial glutathione via redox-sensitive sites of ANT [55]. It has been reported that a 3-thia fatty acid (3-thiastearic acid), with a chain length comparable to that of TTA, inhibits ANT activity accompanied by dissipation of $\Delta\psi$ [36]. Hence, it is possible that TTA mediates mitochondrial permeabilization both directly, e.g., by interacting with ANT, or indirectly, by modulating mitochondrial glutathione. Moreover, TTA was found to uncouple mitochondria in rat hepatocytes, and the reduction of the proton electrochemical potential (Δp) was attributed to lowering of $\Delta\psi$ and not the pH gradient (ΔpH) (our unpublished data). We therefore suggest that TTA-mediated uncoupling might involve an electrogenic ion transport system in the mitochondrial inner membrane, e.g., a metal-ion uniporter. A candidate could be UCP-2, which was significantly upregulated in the liver of TTA-treated rats; however, it is yet to be clarified if UCP-2 can facilitate pH neutral ion transport across the inner membrane.

Induction of cell death by cAMP in the leukemia cell line IPC has been well characterized [49]. In the present study, we show that 8CPT-cAMP treatment slightly changes the mitochondrial glutathione redox status without altering the level of total free glutathione. Whether this is crucial for 8CPT-cAMP-induced apoptosis, or rather is a secondary consequence, should be considered. Furthermore, mitochondria seemed to aggregate after 8CPT-cAMP treatment (Figure 6A), and similar observations have been reported for other types of apoptosis [50]. The functional aspects of such organelle redistribution remain elusive.

We conclude that TTA induces apoptotic pathway(s) involving early mitochondrial alterations, including modulation of the mitochondrial glutathione status.

Significance

Mitochondria have been proposed as targets for anticancer chemotherapy [56]. This is based on the ability of some compounds that induce mitochondrial permeabilization to overcome apoptosis resistance in cancer cells with protective abnormalities in their apoptotic machinery. Accordingly, it was interesting to observe that TTA treatment gave an early mitochondrial response and partly overcame Bcl-2-mediated apoptosis inhibition. Furthermore, focusing on mitochondrial regulation of apoptosis, the mitochondrial redox status has been proposed as a contributing factor. This understanding is supported by the results showing that TTA selectively modulates glutathione level and redox status in mitochondria early in the induction of apoptosis.

Experimental Procedures

Cell Culture and Treatment

The IPC-81 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated horse serum. Generation and characterization of the transfected IPC-81 Bcl-2 and IPC-81 ICER subclones are described elsewhere [25, 27]. For experimental purposes, the cells were seeded at a density of 0.4×10^6 cells/ml. The cell-permeable cAMP analog 8CPT-cAMP was used to study cAMP-mediated effects. TTA was prepared at the Department of Chemistry, University of Bergen, Norway as previously described [57]. TTA was dissolved in 0.1 M NaOH before addition to horse serum or essential fatty acid free BSA as described in [46]. The level of apoptosis was determined by microscopic cell surface examination and fluorescence analysis after staining with the DNA-specific dyes daunorubicin (5–10 μM) or Hoechst 33342 (10 μg/ml) in 4% paraformaldehyde. Apoptotic cells were easily discriminated from non-apoptotic cells by the appearance of multiple surface buds, chromatin condensation, and nuclear fragmentation. The pan-caspase antagonist zVAD-fmk (BACHEM AG, Bubendorf, Switzerland) was used at a final concentration of 100 μM.

Preparation of Cellular Fractions

Cell cultures were harvested by centrifugation and washed in PBS before they were resuspended in a small volume (1–1.5 ml) of H buffer (50 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$, and 0.25 M sucrose, pH 7.5). The cells were homogenized using a ball-bearing Cell-Cracker [58] (EMBL, Heidelberg, Germany). Cell debris was removed by centrifugation at 700 $\times$ g for 10 min (4°C). The resulting suspension was centrifuged at 13,000 rpm (14,500 $\times$ g) for 15 min (4°C) in a Beckman Optima tabletop ultracentrifuge with TLS-55 swinging bucket rotor for isolation of mitochondria (pellet). The supernatant was centrifuged at 33,000 rpm (93,300 $\times$ g) for 30 min (4°C) for preparation of cytosolic fraction (supernatant). The protein content of the fractions was measured with Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA), using BSA dissolved in distilled water as standard.

Cytochrome c Immunoassay

Cytochrome c was determined using a rat/mouse cytochrome c immunoassay (R&D Systems, Inc., Minneapolis, MN).

Caspase-3 Activity

A colorimetric assay (R&D Systems) with DEVD substrate peptide conjugated to p-nitroanaline was used to determine the enzymatic activity of the caspase-3 class of proteases.

Determination of Total (GSSG + GSH) and Nonoxidized (GSH) Free Glutathione

For determination of free glutathione, cellular fractions were extracted with equal volumes of 5% sulfosalicylic acid with 50 μM dithioerythritol before they were maintained at −80°C. Sample preparation and HPLC analysis were performed according to the method described by Svardal et al. [59]. We both assessed the amount of GSH and the total amount of glutathione equivalents present as either GSH or oxidized glutathione-disulfide (GSSG). GSSG contains two equivalents of glutathione. Thus, total glutathione, or GSH + GSSG, means the total amount of glutathione equivalents, and the ratio GSH/(GSH + GSSG) reflects the fraction of total glutathione being nonoxidized.

Western Analysis

Protein from extracts were separated by SDS-PAGE and transferred to nitrocellulose membrane (Hybond ECL; Amersham) according to standard techniques. Blots were probed with rabbit polyclonal antibodies against caspase-3 (sc-7148; Santa Cruz Biotechnology Inc.) and PARP (sc-7150; Santa Cruz Biotechnology Inc.), and goat horseradish peroxidase-conjugated anti-rabbit antibody (Bio-Rad Laboratories). Equal loading and transfer were confirmed by probing with mouse monoclonal anti-actin antibody (clone ac-40; Sigma-Aldrich Co).

Cellular Imaging

Cells were loaded with 250 or 500 nM CellTracker green (5-chloromethylfluorscein diacetate, CMFDA) and 800 nM Mitotracker Red CM-H_2Xros (MTR) (Molecular Probes, Inc., Eugene, OR), according to the manufacturer's recommendations. Following loading, the medium was removed by centrifugation, and coverslips were mounted with fresh tissue culture medium. The prepared cells were studied immediately with a BIO-RAD MRC-1000 Laser Scanning Confocal imaging system connected to a Zeiss Axiovert 100 microscope. Independent simultaneous scans of red (ex. 568 nm, em. 585 nm) and green (ex. 488 nm, em. 522/35 nm) fluorescence were made to identify MTR and CMFDA staining, respectively.

Determination of $\Delta\psi$

Cells were incubated for 10 min with 10 μg/ml of the cationic carbocyanine dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1; Molecular Probes, Inc.). Negative control cells were simultaneously treated with the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 10 μM). The cells were washed twice in saline prior to analysis in a Coulter EPICS XL-MCL Flow Cytometer. Depolarization of $\Delta\psi$ was indicated by a decrease in red fluorescence (590 nm).

Statsistics

The results are obtained from two to five separate experiments for each parameter and are presented as mean ± SD. If measurements could not be performed in duplicate or triplicate samples, the experiment was repeated at least three times. Student's t test (two group analysis) and ANOVA test with Dunnett multiple comparison post test were applied to test for equality of means of each group compared with the control at the significance level $\alpha = 0.05$.

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Note Added in Proof

The data referred to as "our unpublished data" are now in press: Grav, H.J., Tronstad, K.J., Gudbrandsen, O.A., Berge, K., Fladmark, K.E., Martinsen, T.C., Wergedahl, H., and Berge, R.K. (2003). Changed energy state and increased mitochondrial β-oxidation rate in liver of rats associated with lowered proton electrochemical potential and stimulated UCP-2 expression. Evidence for PPARα independent induction of UCP2 expression. J. Biol. Chem., in press.