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tBid induces alterations of mitochondrial fatty acid oxidation flux by malonyl-CoA-independent inhibition of carnitine palmitoyltransferase-1

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Abstract

Recent studies suggest a close relationship between cell metabolism and apoptosis. We have evaluated changes in lipid metabolism on permeabilized hepatocytes treated with truncated Bid (tBid) in the presence of caspase inhibitors and exogenous cytochrome *c*. The measurement of β-oxidation flux by labeled palmitate demonstrates that tBid inhibits β-oxidation, thereby resulting in the accumulation of palmitoyl-coenzyme A (CoA) and depletion of acetyl-carnitine and acylcarnitines, which is pathognomonic for inhibition of carnitine palmitoyltransferase-1 (CPT-1). We also show that tBid decreases CPT-1 activity by a mechanism independent of both malonyl-CoA, the key inhibitory molecule of CPT-1, and Bak and/or Bax, but dependent on cardiolipin decrease. Overexpression of Bcl-2, which is able to interact with CPT-1, counteracts the effects exerted by tBid on β-oxidation. The unexpected role of tBid in the regulation of lipid β-oxidation suggests a model in which tBid-induced metabolic decline leads to the accumulation of toxic lipid metabolites such as palmitoyl-CoA, which might become participants in the apoptotic pathway.

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Abbreviations: CPT-1, carnitine palmitoyltransferase-1; CPT-2, carnitine palmitoyltransferase-2; CoA, coenzyme A; tBid, truncated Bid; $\Delta\Psi_m$, mitochondrial transmembrane potential; siRNA, small interfering RNA; zVAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Aspfluoromethyl ketone; PMSF, phenyl methyl sulfonyl fluoride; TMRE, tetramethylrhodamine ethyl ester; COX4, cytochrome *c* oxidase polypeptide IV; BMH, bismaleimidohexane

Introduction

As a powerhouse of energy, the mitochondria serve to produce cellular energy.[1] During this process, acetyl-coenzyme A (CoA), the breakdown product of fatty acids and glucose, is passed through the Kreb's cycle to generate electrons. These, in turn, are transferred to the proteins of the respiratory chain in the inner mitochondrial membrane, generating the mitochondrial transmembrane potential ($\Delta\Psi_m$), which results from an asymmetric distribution of protons on both sides of the inner mitochondrial membrane. The $\Delta\Psi_m$ creates an electrochemical gradient, which is used to produce ATP.

In addition to serving as the main intracellular source of energy of the cell, mitochondria regulate several cellular processes that are linked to apoptosis.[2] They are indeed the storage site for a number of soluble factors that mediate apoptosis, including cytochrome *c*, certain procaspases and apoptosis-inducing factor.[3] Mitochondria can activate apoptosis by releasing these factors from their intermembrane space into the cytoplasm. The relocalization of these mitochondrial proteins results in the direct activation of caspases, the neutralization of cytosolic caspase inhibitors and the activation of nucleases.

Recent studies suggest a more intimate integration of cellular energy metabolism and apoptosis. Changes in either glycolytic or lipid metabolism are sufficient to cause commitment to programmed cell death and can directly induce decisive disruption of mitochondrial function and collapse of the $\Delta\Psi_m$. For example, cellular glycolysis may be restricted by decreasing the glucose level in the medium. Severe restrictions in glycolysis result in cell death also in the presence of prosurvival growth factors. Surprisingly, death as a result of reducing the concentration of glucose proceeds via apoptosis because it induces the activation of Bid, the release of cytochrome *c* and the activation of caspases.[4] Again, palmitate and similar saturated long-chain acyl fatty acids are also known to induce apoptosis via a mechanism that is not completely elucidated.[5] It has been described that palmitate can determine the generation of ceramide, a proapoptotic second messenger, via a pathway that is stimulated by pharmacological inhibition of carnitine palmitoyltransferase-1 (CPT-1).[6]

Moreover, it is becoming increasingly clear that not only cellular metabolism plays an important regulatory role in the control of programmed cell death but also elements of the apoptotic machinery can affect the cellular metabolism. Interestingly, several papers have demonstrated that truncated Bid (tBid) elicits dissociation, from mitochondria, of hexokinases (HK), enzymes catalyzing the phosphorylation of glucose, the first committed step of glucose metabolism.[4] Activated serine/threonine kinase Akt/protein kinase B hinders the proapoptotic activity of tBid by coupling of glucose

metabolism to oxidative phosphorylation and by regulating PT pore opening favoring the HK interaction with the outer mitochondrial membrane.[4–7]

Less is known about the connection between tBid-mediated mitochondrial dysfunction and fatty acid metabolism. In particular, it is uncertain whether tBid-induced mitochondrial β-oxidation perturbations result exclusively from the loss of cytochrome *c* or whether other irreversible events occur. Finally, there are no data about the appearance, in cells exposed to tBid, of specific fatty acid metabolites able to act as endogenous stress signals on mitochondria, thus becoming integral participants in the apoptotic pathway.

To gain further insight into the mechanism of the effect of tBid on fatty acid metabolism in the present study, we applied permeabilized cell techniques to assess mitochondrial function in the hepatocytes after tBid, caspase inhibitors and exogenous cytochrome *c* addition to the assay medium. Studying intact cells provides only limited information about mitochondrial function because it is difficult to control the extra mitochondrial medium and the mitochondrial functional state.[8,9] Permeabilization of the plasma membrane for low molecular weight solutes allows the investigation of mitochondrial processes under precisely controlled conditions *in situ*, where mitochondrial interaction with intracellular structures is largely preserved.[10]

Early events of apoptosis that have been demonstrated in other cell systems have been reproduced herein.[11,12] Specifically, we examined the temporal relationship between the loss of cytochrome *c* and the loss of mitochondrial membrane potential after tBid addition to permeabilized cells. We confirmed that the tBid-induced release of cytochrome *c* from mitochondrial intermembrane space can proceed in a caspase-independent manner, and that $\Delta\Psi_m$ could be maintained, in the absence of caspase activation, by exogenous addition of cytochrome *c*. Although the apparent normality of mitochondrial functions in terms of metabolic capacity, measurement of several mitochondrial parameters related to fatty acid β-oxidation indicated that tBid caused β-oxidation inhibition via a blockade of the enzyme CPT-1 and an increase of palmitoyl-CoA. Furthermore, we demonstrated that the observed changes in CPT-1 activity were independent from malonyl-CoA, the key inhibitory molecule of CPT-1. Hence, a novel mechanism has to be involved in the tBid modulatory effect on CPT-1 activity, and is probably related to the capacity of tBid to affect the lipid composition of mitochondrial membranes. Indeed, our findings showed a decrease in cardiolipin level in mitochondria after tBid addition. This event, inducing a change in the lipid environment of mitochondrial membranes, may influence the activity of CPT-1 extremely sensitive to the lipid constituents of the microdomain in which it resides.[13,14]

To test whether the tBid-mediated effects on fatty acid metabolism and on CPT-1 activity requires proapoptotic members of the Bcl-2 family, such as Bax and Bak, suppression of gene expression by small interfering RNA (siRNA) was utilized to inhibit Bak and Bax expression in cultured cells. The results obtained have clearly demonstrated that their downregulation did not affect significantly the ability of tBid to reduce β-oxidation flux.

As Bcl-2 physically interacts with CPT-1 and tBid,[15,16] we next examined whether the Bcl-2 overexpression prevented the effect of tBid on CPT-1 activity. Interestingly, Bcl-2-overexpressing cells showed a normal pattern of fatty acid β-oxidation also in the presence of tBid. Thus, Bcl-2 antagonizes both the proapoptotic activity and the metabolic inhibitory capacity of tBid. On the other hand, tBid was not able to create complexes with CPT-1 as demonstrated by co-immunoprecipitation assay. Therefore, the CPT-1 inhibition mediated by tBid is most likely a result of an indirect rather than a direct effect of the proapoptotic factor on the enzyme.

Irrespective to the theoretical possibilities resulting from our experiments, it appears clear that (1) tBid, inhibiting β-oxidation flux, can induce the generation of potentially toxic fatty acid metabolites, such as palmitoyl-CoA, able to act as endogenous stress signals on mitochondria,[17,18] and that (2) tBid-induced metabolic alterations can be prevented by Bcl-2 overexpression.

Thus, for the first time we have shown that during apoptosis induced by tBid, mitochondria experience a progressive dysfunction that involves not only glucose metabolism, as already demonstrated, but also β-oxidation flux of long-chain fatty acid, and that compels the cell to the 'point-of-no-return' of the death program.

Results

Inhibition of tBid-dependent mitochondrial membrane potential decrease by caspase inhibitors and cytochrome *c* in permeabilized hepatocytes

The activation of Bid and its translocation as tBid to mitochondrial membranes, attracted by the mitochondrion-specific lipid cardiolipin, is critical in the induction of apoptosis in certain types of cells such as hepatocytes.[19] However, in the absence of the downstream caspase activities after cytochrome *c* release, Bid seems to be not able to affect both cell viability and mitochondrial functional integrity.[20]

For the purposes of further studying the contribution of tBid in the regulation of mitochondrial function, we used permeabilized hepatocytes where the time-consuming and potentially damaging mitochondrial purification process was not necessary. In fact, the aim of the permeabilization procedure was to allow access of tBid and, where requested, cytochrome *c* to the mitochondrial compartment while preserving intact mitochondria. Moreover this model has the advantage of preserving mitochondria in their normal intracellular location relative to other organelles and cytoskeleton proteins, and of being available for study within seconds of disrupting the plasma membrane. The degree of permeabilization was assessed by evaluating the release of marker enzymes from the cytosol (lactate dehydrogenase) and mitochondrial matrix (citrate synthase). About 80% of lactate dehydrogenase was released in the supernatant with digitonin at 40 μg/ml, while 95% of the cells became permeable to Trypan blue (Figure 1). This digitonin concentration did not produce mitochondrial permeabilization, since all citrate synthase activity remained in the pellet, thus indicating that the mitochondria were intact (Figure 1).

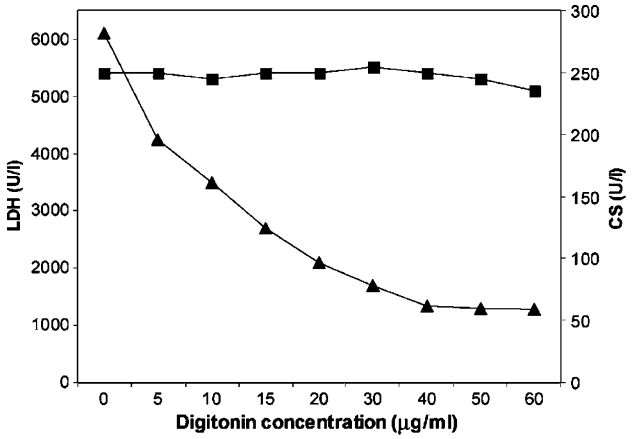

Figure 1 Release of lactate dehydrogenase and citrate synthase during digitonin permeabilization of hepatocytes. Cells were suspended in a buffer containing 220 mM mannitol, 68 mM sucrose, 10 mM Hepes (pH 7.4), 70 mM KCl, 1 mM EGTA, 1 mM PMSF and 2 μM aprotinin. The experiments were carried out to choose the minimum amount of digitonin capable of selectively permeabilizing the plasma membrane within 5 min without affecting mitochondrial integrity. Lactate dehydrogenase (▲) and citrate synthase (■) activities were measured in the cell pellet after washing with PBS

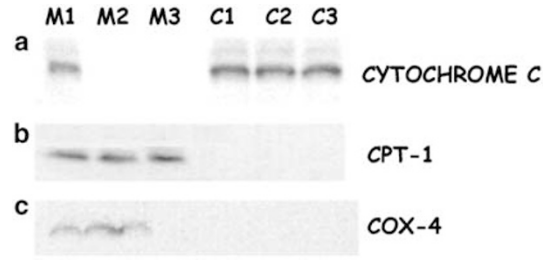

Figure 2 Outer membrane permeability and loss of cytochrome *c* in mitochondria from digitonin-permeabilized hepatocytes treated with tBid. (**a**) The mitochondrial pellet (M) and cytosolic fractions (C) from hepatocytes after 30 min incubation at 37°C w/wo tBid (20 ng/ml), in the presence or absence of zVAD-fmk (zVAD; 100 μM), were probed with cytochrome *c* antibody ($\sim$15 kDa). (**b**) CPT-1 levels in mitochondria from digitonin-permeabilized hepatocytes treated or untreated with tBid. The experiment was performed as in panel a, except that the fractions were probed with CPT-1 antibody ($\sim$88 kDa). (**c**) Mitochondrial and cytosolic fractions from treated or untreated cells probed with COX4 Antibody ($\sim$17 kDa). M: mitochondrial fraction – M1: control; M2: tBid; M3: tBid + zVAD; C: cytosolic fraction – C1: control; C2: tBid; C3: tBid + zVAD

To ascertain whether the mitochondria from hepatocytes treated with tBid had depleted cytochrome *c* independently of caspase activation, we isolated mitochondria from permeabilized cells, and assessed the amount of cytochrome *c* in the mitochondrial pellet (M) and supernatant (C) fractions. Nearly all of the cytochrome *c* was released from mitochondria isolated from the cells following tBid addition to the incubation mixture both in the presence or in the absence of *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk), a general caspase inhibitor (Figure 2a).

Then, we determined whether tBid could induce loss of $\Delta\Psi_m$ in permeabilized cells, in the presence or in the absence of exogenous cytochrome *c* added to the incubation mixture. Consistent with previous results, addition of cytochrome *c* restored $\Delta\Psi_m$ in zVAD-fmk- and tBid-treated

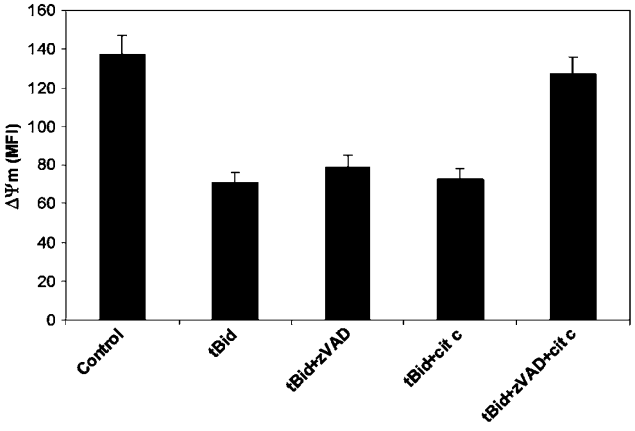

Figure 3 Cytochrome *c* restores $\Delta\Psi_m$ after tBid addition only in the presence of caspase inhibitor. Permeabilized hepatocytes (10^6) were incubated with or without tBid (20 ng/ml), in the presence or absence of zVAD-fmk (zVAD; 100 μM) and/or cytochrome *c* (cit c; 100 μM) plus TMRE at 37°C for 30 min with succinate as substrate. Cells were analyzed for $\Delta\Psi_m$ by flow cytometry; the mean fluorescence intensity (MFI) values for cells treated with FCCP (trifluorocarbonylcyanide phenylhydrazone) to dissipate $\Delta\Psi_m$ was set as 0

cells, while in the absence of anticaspase inhibitor, tBid induced a loss of $\Delta\Psi_m$, which was not restored by cytochrome *c* addition (Figure 3).

These data strongly support the conclusion that caspase functions were required for the onset of apoptotic events and of mitochondrial dysfunction as depicted by the dramatically $\Delta\Psi_m$ decrease upon caspase activation. Without caspase activation, most mitochondria appeared to remain functional after the permeabilization of the outer mitochondrial membrane if cytochrome *c* was exogenously added to the incubation mixture.

Inhibition of fatty acid oxidation in hepatocytes by tBid

The activation of the proapoptotic Bcl-2 members, such as Bid, in the presence of caspase inhibitors can permeabilize the mitochondrial outer membrane without disrupting $\Delta\Psi_m$. Since mitochondrial functions including protein import, ATP generation, and lipid biogenesis depend on the maintenance of $\Delta\Psi_m$, it is likely that tBid does not affect the mitochondrial metabolic activity if cytochrome *c* is sufficiently available in the cell microenvironment.[21]

To evaluate whether the translocation of tBid to the mitochondria might induce changes in fatty acid oxidation, we treated hepatocytes with tBid in the presence of the caspase inhibitor, zVAD-fmk, and cytochrome *c*.

At first, we examined in our system the rate of β-oxidation flux of labeled palmitate, calculated as total acid-soluble radioactivity, as $^{14}CO_2$ release, or as total ^{14}C-labeled products (i.e. the sum of acid-soluble radioactivity and of $^{14}CO_2$ release). Since the permeabilization process led to an increased availability of palmitate and carnitine to the mitochondria, a high production of acid-soluble radioactive metabolites was demonstrated in permeabilized untreated hepatocytes after palmitate addition (Figure 4). The accumulation of these

metabolites was linear for at least 90 min. We also evaluated the contribution of peroxisomal β-oxidation in our experiments. The data present in Figure 4 clearly demonstrates that the oxidation of substrate was significantly inhibited by the presence of inhibitors of the respiratory chain. Thus, the contribution of peroxisomes to the oxidation of palmitate under our experimental conditions is minimal, since peroxisomal activity is independent of the respiratory chain. After treatment with tBid, the rate of oxidation of palmitate slowed down during the initial 30 min (Figure 5). The maximum difference in oxidation rate between treated and untreated hepatocytes was reached after 1 h (three-fold difference). Figure 5 also shows that the oxidation of octanoate is not depressed at all by tBid treatment. It is well known that octanoate, a medium-chain fatty acid, is transported into the mitochondria independently of carnitine, while palmitate, a long-chain fatty acid, is imported into the mitochondria by a carnitine-dependent transport. Thus, the results obtained may indicate that the tBid treatment depresses fatty acid oxidation by means of the downregulation of carnitine-dependent transport of fatty acid into mitochondria.

To determine whether tBid also influenced pyruvate flux through pyruvate dehydrogenase (PDH), permeabilized cells were incubated in the presence of labeled pyruvate, and its conversion into $^{14}CO_2$ was analyzed. These experiments showed that tBid caused an increase in $^{14}CO_2$ production rather than a decrease when the cells were incubated with pyruvate (331.5 *versus* 391.1 nmol CO_2/30 min/mg protein released from control and tBid-treated cells, respectively).

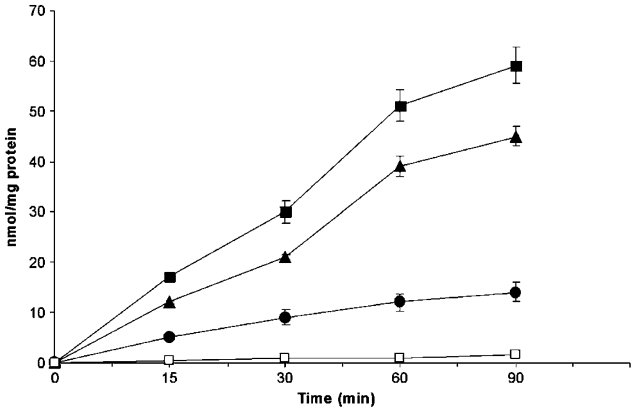

Figure 4 β-Oxidation flux in permeabilized hepatocytes. Incubations were performed and products analyzed as described in the 'Materials and Methods' section. Results are means $\pm$ S.D. for three to five incubations. Symbols: ■, total ^{14}C-labeled products; ▲, total acid-soluble radioactivity; ●, $^{14}CO_2$ release; □, total ^{14}C-labeled products in the presence of a mixture of rotenone (5 mg/l), antimycin A (5 mg/l) and sodium cyanide (1 mM)

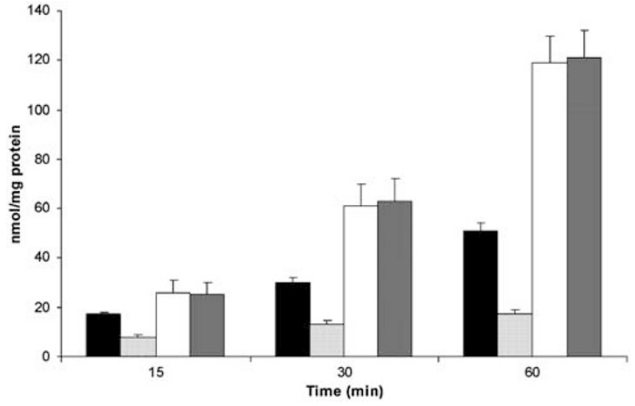

Figure 5 tBid-mediated inhibition of β-oxidation flux in hepatocytes. Hepatocytes were incubated with [1-^{14}C]palmitate or [1-^{14}C]octanoate in the presence or absence of tBid (20 ng/ml) plus zVAD-fmk (100 μM) and cytochrome *c* (100 μM) at the indicated times. Total ^{14}C-labeled products were measured as described in the 'Materials and Methods' section. Values are means $\pm$ S.D. from three parallel experiments. Symbols: ■, palmitate; ▨, palmitate + tBid; □, octonoate; ▩, octonoate + tBid

β-Oxidation intermediate profiles in tBid-treated cells

To verify the possibility that a deficiency of the mitochondrial carnitine system might occur following tBid treatment, we analyzed the pattern of β-oxidation intermediates in cells incubated in the presence of labeled palmitate. To select the incubation time for determining the pattern of intermediates, we carried out preliminarily a time-course incubation and analyzed the acyl-CoA and acylcarnitine esters generated. Preliminary results showed that, under the experimental conditions applied, a typical intermediate profile is achieved rapidly and maintained for up to 2 h. Thus, an incubation time of 60 min was chosen and used throughout the rest of the study. In permeabilized control cells, only traces of palmitoyl-CoA esters could be detected, while acylcarnitine esters were easily identified, showing a typical pattern of intermediates. In particular, acetylcarnitine and palmitoyl-carnitine were the main carnitine esters detected in control cells. The other intermediates were present only in traces and are not reported in Table 1. In tBid-treated cells, the absolute amount of palmitoyl-CoA detected was four times the average amount of palmitoyl-CoA detected in the control cells. On the other hand, it was not possible to identify or quantify most of acylcarnitines intermediates. Only acetylcarnitine production was detectable at low level. The results of quantitative palmitoyl-CoA and acylcarnitine profiles accumulating in the reaction mixture after tBid treatment compared with controls were summarized in Table 1.

Taken together, these findings provide further corroboration of the puzzling observation that tBid induces alterations of the mitochondrial carnitine-dependent transport of fatty acids.

Table 1 Amount of ^{14}C-labeled palmitoyl-CoA, acetylcarnitine and palmitoyl-carnitine generated during the oxidation of [U-^{14}C]palmitate by hepatocytes in the presence or in the absence of tBid (20 μg/ml), cytochrome *c* (100 μM) and zVAD-fmk (100 μM)

	Control	tBid
Palmitoyl-CoA (pmol/h/mg protein)	10 $\pm$ 1	43 $\pm$ 1
Acetylcarnitine (nmol/h/mg protein)	33.7 $\pm$ 7	15.9 $\pm$ 3
Palmitoylcarnitine (nmol/h/mg protein)	1.45 $\pm$ 0.6	0.4 $\pm$ 0.1

tBid inhibition of CPT-1: the role of malonyl-CoA

One possible target for tBid action might be CPT-1, the enzyme involved in the synthesis of palmitoyl-carnitine from activated palmitate. CPT-1 catalyzes the pace-setting step of long-chain fatty acid import into the mitochondrial matrix. The other components of the carnitine system, namely carnitine/acylcarnitine translocase and carnitine palmitoyltransferase-2 (CPT-2), are generally not considered to play a significant regulatory role in the translocation of long-chain fatty acid into the mitochondrial matrix.

An assay was performed to test CPT-1 activity on permeabilized primary human hepatocytes to further evaluate the mechanism underpinning the observation that tBid addition to cells influenced mitochondrial fatty acid β-oxidation flux. As shown in Figure 6, CPT-1 activity was decreased by exposure of permeabilized hepatocytes to tBid, thus demonstrating a correlation between decreased enzyme activity and diminished palmitate utilization. To exclude enzyme degradation and/or leakage at the outer mitochondrial membrane, a quantitative analysis of CPT-1 was carried out on isolated mitochondria by immunoblotting. No evidence of variations in the amount of mitochondrial CPT-1 was demonstrated (Figure 2b).

Since it has been shown that CPT-1 is reversibly inhibited by malonyl-CoA, it was conceivable that tBid-induced inhibition of long-chain fatty acid oxidation was mediated by an increased intracellular malonyl-CoA level. On the other hand, because the permeabilization process of the hepatocytes led to a substantial dilution of cytosolic components including malonyl-CoA, it was unlikely that the intracellular level of malonyl-CoA could reach a value capable of inhibiting CPT-1 activity.

To understand whether the mechanism involved in the tBid-induced inhibition of CPT-1 was malonyl-CoA dependent or independent, enzyme activity was determined by a procedure that eliminated any possible interference of malonyl-CoA. In such an assay, permeabilized cells, once treated with tBid, were extensively washed to achieve complete removal of malonyl-CoA. Determination of the enzyme activity was then performed after a lag period of 15 min, so that any conformational constraint of CPT-1, due to previous binding with malonyl-CoA, disappeared. Finally, the malonyl-CoA level was evaluated in parallel cultures undergone the same procedure.

The use of this method confirmed that the inhibition of CPT-1 by tBid treatment is exerted by a malonyl-CoA-independent mechanism. Indeed, no detectable amounts of malonyl-CoA were found in cells processed as described. In this respect, it is noteworthy that the negative effect of tBid on the catalytic efficiency of CPT-1 was identical to that in the previous experiments.

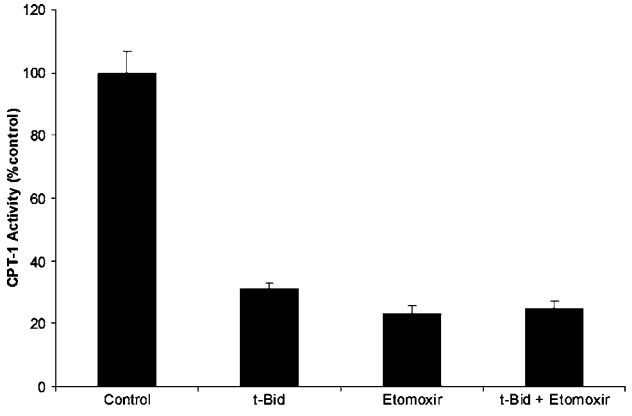

Figure 6 Inhibitory effect of tBid on CPT-1 activity in permeabilized hepatocytes. Digitonin-permeabilized cells were preincubated for 30 min in the absence or in the presence of tBid (20 ng/ml) plus zVAD-fmk (100 μM) and cytochrome *c* (100 μM). Palmitoyl-carnitine formation from labeled L-carnitine and palmitoyl-CoA was measured after cell incubation for 5 min with or without etomoxir (100 μmol/l), an irreversible CPT-1 inhibitor. Results correspond to seven different hepatocytes preparations and are given as means $\pm$ S.D. Significantly different *versus* incubations with no additions: $P<0.01$

tBid affects the cardiolipin levels in mitochondrial membranes

Previous results have shown that the outer membrane CPT-1 and the inner membrane CACT and CPT-2 are enriched in the mitochondrial contact sites, suggesting an organization within the contact site that facilitates the carnitine-dependent transport of fatty acyl groups across the mitochondrial membrane. Contact sites are purported to have a unique lipid environment that is rich in cardiolipin. This lipid is associated, probably with a regulatory function, with numerous mitochondrial transporters such as the adenine nucleotide translocator (ANT). Interestingly, recent papers have demonstrated both that the targeting of tBid to mitochondria depends upon the presence of the cardiolipin and that tBid localizes specifically to the mitochondrial contact sites rich in cardiolipin.[22–24]

To evaluate whether tBid was capable to modify the lipid composition of the mitochondrial membranes, thus affecting fatty acid import machinery, we analyzed the mitochondrial cardiolipin content by HPLC. The cardiolipin concentration was calculated in reference to internal standard, tetrastearoyl-cardiolipin, a synthetic analog uncommon in biological specimens. The method applied allowed a measure of cardiolipin concentration with a quantitation limit of 0.5 nmol. As depicted in Figure 7, exposure of mitochondria to tBid resulted in a decrease of cardiolipin level compared with untreated mitochondria.

tBid-induced alterations of fatty acid metabolism do not require functions of the proapoptotic proteins Bax and Bak and are inhibited by Bcl-2

Well-known mediators of mitochondria damage include the proapoptosis members of the Bcl-2 family, Bax and Bak, which may self-oligomerize in mitochondria outer membrane to permeabilize it.[25] In a mouse model, knockout of both Bax and Bak conferred resistance to normal apoptosis in animal development and in experimental apoptosis induced by many apoptosis-inducing agents.[26]

Thus, the ability of Bak and Bax to act as agonist of tBid in inducing modifications of mitochondrial fatty acid flux was also investigated by using siRNA-mediated knockdown. Hepatocytes were transfected either with a control siRNA (a random

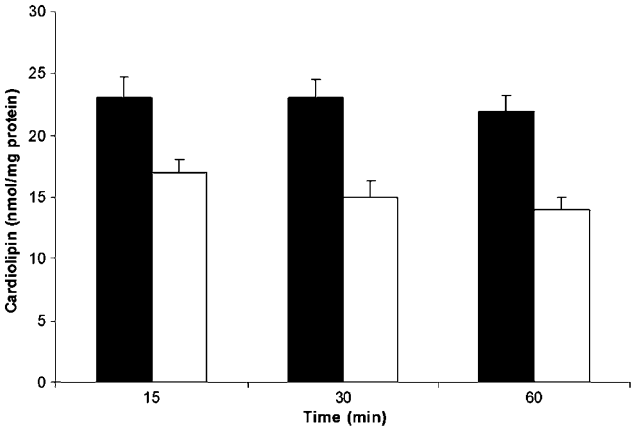

Figure 7 Loss in the cardiolipin content in mitochondria of hepatocytes incubated with tBid. Hepatocytes were incubated at 37°C for different times in the absence (control) (■) or presence (□) of tBid (20 ng/ml) plus zVAD-fmk (100 μM) and cytochrome *c* (100 μM). Cardiolipin content was determined as described in 'Materials and Methods'. All values are expressed as mean ± S.D. of five separate experiments

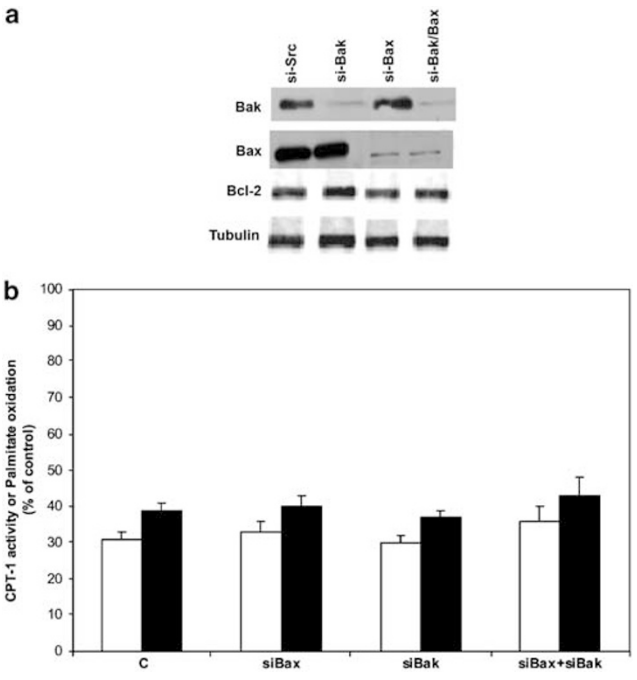

Figure 8 Suppression of Bax and/or Bak gene expression by specific si-RNAs fails to inhibit the effects of tBid on CPT-1 activity and β-oxidation flux. (**a**) Gene-specific si-RNAs (si-Bak and/or si-Bax) or the control si-RNAs (si-Src) were added to the media using lipophilic transfection-enhancing reagent. Cells were harvested after 48 h and immunoblot analyses were performed using Bcl-2-, Bax- and Bak-specific antibodies. The blots were reprobed with antibody against tubulin to confirm equal protein loading. Control si-RNAs had no effect; however, Bax and Bak si-RNAs effectively inhibited expression of the specific genes by > 93% (three independent experiments). No downregulation of Bcl-2 expression was observed. (**b**) Control cells (C) and cells transfected with Bax and/or Bak si-RNAs were permeabilized and preincubated for 30 min in the presence of tBid (20 ng/ml) plus zVAD-fmk (100 μM) and cytochrome *c* (100 μM). Subsequently, the rate of CPT-1 activity (□) as well as palmitate oxidation (■) was determined (see Materials and Methods). The values are expressed as the percentage of the controls without tBid. Results correspond to seven different hepatocytes preparations and are given as means ± S.D.

scrambled sequence; si-Scr) or with gene-specific siRNAs targeted against Bax (si-Bax), and/ or Bak (si-Bak). Cells were analyzed at 48 and 72 h post-transfection for protein expression by immunoblotting (Figure 8). While both si-Bax and si-Bak inhibited specific protein expression, si-Scr had no significant effect on expression of Bax or Bak. si-Bax downregulated Bax, but no such effect was observed on expression of either Bcl-2 or Bak. Similarly, si-Bak was specific for Bak as no downregulation of Bax, or Bcl-2 proteins was observed.

Since siRNA has been shown to result in interferon (IFN) gene induction in some cells,[27] transfected cells were also analyzed for two different IFN-stimulated genes (ISGs), signal transducer and activator of transcription 1 (STAT-1) and ISG15 (p15).[28] Compared to controls, the gene-specific siRNAs utilized in this study did not induce STAT-1 or ISG15 protein expression in the cells (data not shown).

Knockdown of Bax or Bak did not affect the ability of tBid to modulate negatively the mitochondrial β-oxidation capacity via CPT-1 inhibition, suggesting that this effect of tBid is not dependent on Bax or Bak alone. Also when both Bax and Bak were subjected to knockdown, the capacity of tBid to induce CPT-1 inhibition was not decreased (Figure 8).

We next determined whether the Bcl-2 overexpression prevented the effect of tBid on lipid metabolism. Interestingly, Bcl-2-overexpressing cells showed a normal pattern of fatty acid β-oxidation and CPT-1 activity also in the presence of tBid (Figure 9). Together, these results suggest that, in addition to preventing the apoptotic pathway, Bcl-2 may antagonize tBid-induced metabolic dysfunctions of mitochondria.

tBid does not co-immunoprecipitate with CPT-1

Our results suggested that after insertion into mitochondrial membranes tBid inhibited CPT-1 activity. One hypothesis about the underlying mechanism was that tBid might directly interact with CPT-1, resulting in conformational changes in this molecule that prevented its enzymatic catalysis. To test this possibility, we examined the interactions between CPT-1 and tBid by co-immunoprecipitation. In this experiment, cells extracts were subjected to immunoprecipitation with CPT-1 antibodies. The immunoprecipitates were analyzed for the presence of tBid. As shown in the Figure 10, tBid was not shown in any of the CPT-1 immunoprecipitates. To demonstrate that the extracts prior to immunoprecipitation did contain tBid, we analyzed the extracts directly by immunoblotting (Figure 10). Clearly, the results suggest that CPT-1 did not co-immunoprecipitate 15-kDa tBid under the experimental conditions applied, although membranous insert of tBid was present.

Discussion

As mitochondria are major players in the cell death decision and in cellular energy metabolism, it is not surprising that proapoptotic factors, such as tBid, interacting with the mitochondria can produce changes in the cellular metabolism.

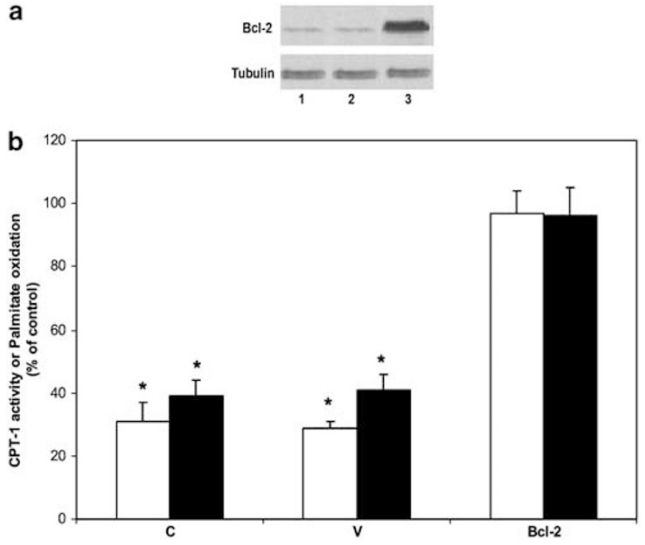

Figure 9 Effects of Bcl-2 overexpression on tBid-induced metabolic alterations. (**a**) Western blot analysis of Bcl-2 and tubulin expression in control cells (1), in empty vector-transfected cells (2) and in Bcl-2-transfected cells (3). (**b**) Control cells (C) and cells transfected with empty vector (V) or Bcl-2 (Bcl-2) were analyzed for CPT-1 activity (□) and palmitate oxidation (■) in the presence of tBid (20 ng/ml) plus zVAD-fmk (100 μM) and cytochrome *c* (100 μM) (see Materials and Methods). The values are expressed as the percentage of the controls without tBid. Results correspond to seven different hepatocytes preparations and are given as means $\pm$ S.D. $^*P < 0.01$

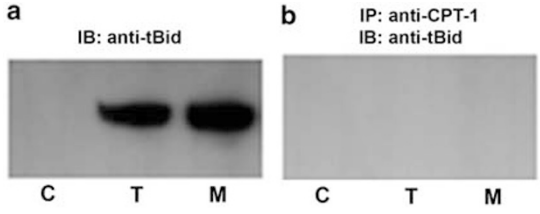

Figure 10 Co-immunoprecipitation analysis of CPT-1 and tBid. Hepatocytes were permeabilized, subjected (T) or not (C) to tBid treatment (see Materials and Methods), and then mitochondria were isolated and incubated with 10 mM BMH crosslinker. The mitochondrial pellets were lysed in RIPA buffer. The extracts were directly used for immunoblot analysis of tBid (IB), or subjected to immunoprecipitation (IP) with anti-CPT-1 antibody followed by detection of tBid in the precipitates by immunoblotting. In another set of experiments, mitochondria (M) were isolated from hepatocytes, incubated with tBid, and then treated with 10 mM BMH. Immunoprecipitation (with anti-CPT-1 antibody) and/or immunoblotting (with anti-tBid antibody) were performed as described before. (**a**) tBid was detected in RIPA extracts prior to immunoprecipitation. In the absence of tBid treatment, no detection was demonstrated (lane C). (**b**) tBid was not shown in any of the precipitates obtained using anti-CPT-1 antibody

In particular, the modulation of lipid oxidation flux by tBid in apoptotic cells deserves more attention not only because mitochondria are a crossroad of the cell lipid trafficking but also because the accumulation of specific fatty acids metabolites may amplify or/and contribute to the apoptotic process.

Indeed, although lipids are often forgotten in the common schemes of apoptosis, they appear to be involved (a) in cell death receptor clustering, (b) in mediating stress-induced apoptosis and/or amplifying cell death signal transduction pathways, (c) in the mitochondrial alterations that accompany apoptosis, or (d) in apoptotic cell recognition and engulfment.[29] This has even led to the proposal of a lipid biostat, which modulates apoptosis induction. In addition, some fatty acids or fatty acid metabolites modulate cell growth and induce apoptosis, some of them displaying properties of potential antitumorigenic compounds.[30,31]

Recent papers suggest an intriguing connection between the accumulation and/or abnormal metabolism of fatty acids and the apoptosis-associated dysfunction of mitochondria. Saturated long-chain fatty acids, such as palmitate, and their cognate CoA-derivatives have been reported to induce mitochondrial membrane permeability transition, to bind to the ANT, to uncouple the respiratory chain and to determine cytochrome *c* release.[18,32] Moreover, it has been reported that palmitoyl-CoA is a precursor of *de novo* synthesis of ceramide, a sphingolipid metabolite important as intracellular mediator of programmed cell death.[33] The generation of the proapoptotic second messenger ceramide is stimulated by inhibition of CPT-1, an enzyme that resides at the outer mitochondrial membrane and catalyzes the synthesis of palmitoylcarnitine from palmitoyl-CoA.[6] CPT-1 together with carnitine/acylcarnitine translocase and CPT-2 regulates the import of long-chain fatty acids into the mitochondrial matrix for β-oxidation. CPT-1 catalyzes the rate-limiting step in this passage and is site for intracellular regulation of fatty acid metabolism, with malonyl-CoA acting as a physiological inhibitor.[34,35] Other studies found a physical association between CPT-1 and Bcl-2 on the outer mitochondrial membrane providing an additional potential link between CPT-1 and the regulation of apoptosis.[15]

Intrigued by these observations, we have explored the rate of β-oxidation as well as the intermediates of β-oxidation and CPT-1 activity in hepatocytes treated with the activated BH3-only protein, tBid. To carry out this experimental design, we developed a procedure for measuring lipid metabolism and CPT-1 activity in digitonin-permeabilized hepatocytes in the presence of caspase inhibitors and exogenous cytochrome *c*. Although tBid causes a rapid release of cytochrome *c*, it does not disrupt significantly $\Delta\Psi_m$ when caspase activity is inhibited. This effect requires a readdition of exogenous cytochrome *c* into the incubation mixture to restore the depleted cytochrome *c* stores. Since mitochondrial functions including protein import, ATP generation, and lipid metabolism depend on the maintenance of $\Delta\Psi$m, it seems that tBid alone is not able to induce mitochondrial dysfunction. On the contrary, measurement of several mitochondrial parameters related to fatty acid β-oxidation indicated that tBid caused β-oxidation inhibition, probably reflecting a blockade of the enzyme carnitine palmitoyl-transferase-1. To test this hypothesis, we used a method for measuring CPT-1 activity in the permeabilized cells. The use of such procedure showed that incubations of hepatocytes with tBid did change CPT-1 activity coordinately with the rate of β-oxidation. Furthermore, we demonstrated that malonyl-CoA, a key inhibitory molecule of CPT-1, was leaked completely out of the permeabilized hepatocytes, demonstrating that a malonyl-CoA was not responsible for the observed changes in CPT-1 activity. Normally, CPT-1 is regulated by at least three mechanisms: (a) variation in enzyme activity assumed to be due to enzyme

synthesis or breakdown; (b) a variable level of malonyl-CoA; and (c) a sensitivity of the enzyme to the composition of the microdomain in which it resides. Usually, the rate of fatty acid β-oxidation is correlated with changes in malonyl-CoA concentration. Only in few cases it has been demonstrated to vary with a malonyl-CoA-independent mechanism.[36] In the present study, we have shown that fatty acid β-oxidation and CPT-1 activity in permeabilized hepatocytes treated with tBid revealed significant changes that are independent of changes in malonyl-CoA levels, and that are too fast to involve enzyme synthesis. Hence, a novel mechanism, probably related to a modified composition of the mitochondrial membrane, has to be involved in the tBid modulatory effect of CPT-1 activity. Traditionally, mitochondrial membranes have been considered static, at least from a biochemical point of view, both in physiological and pathological conditions. This notion has completely changed during the last few years, just for the studies on tBid.

It has been demonstrated that the targeting of the proapoptotic protein tBid to mitochondria depends upon the presence of the mitochondria-specific lipid cardiolipin, which has a specific distribution pattern within mitochondria.[37,38] Cardiolipin is found at high concentrations throughout the inner mitochondrial membrane, including the contact sites between the inner and outer membrane.[37] Immunogold tomography revealed that these contact sites are the preferential targets for association of tBid.[39] Like other membrane-perturbing factors, tBid can alter the lipid composition of mitochondria membrane via its lipid transfer activity that can affect both cardiolipin synthesis, recycling or oxidation, and mitochondrial respiration.[40,41] This would account for the progressive loss of mitochondrial cardiolipin that we found after the addition of tBid to permeabilized cells. The existence of a cause and effect relationship between these two events, cardiolipin loss and decrease of CPT-1 activity, is suggested by the peculiarity of the enzyme insertion in the mitochondrial membrane.

Interestingly, CPT-1 of liver mitochondria is an integral, polytopic protein of the outer membrane that is enriched at contact sites.[42] This topology endows the protein with the potential for modifying the conformation of its catalytic C-domain in response to variations in the membrane environment. Indeed, CPT-1 kinetics are extremely sensitive to the molecular order of the constituent lipids of the membrane environment in which it resides.[13,14] Thus, a possible mechanism for the changes observed in our model might be the altered interaction of CPT-1 with the tBid-modified mitochondrial membrane resulting in a protein conformational change able to affect specifically the acyl-CoA binding site. With respect to this, it is plausible that changes in membrane environment, because of the formation of localized membrane microdomains of distinctive lipid composition due to the action of tBid, would induce altered activity of CPT-1.[14]

Consistent with these observations, our experiments have shown that there is no physical interaction of CPT-1 and tBid as demonstrated by co-immunoprecipitation assay. Thus, the modulation of CPT-1 activity by tBid is most likely a result of an indirect rather than a direct effect of the molecule on the enzyme. Moreover, despite the recognized role for Bax/Bak in inducing loss of mitochondrial integrity, both factors did not show any agonist or antagonist effect on β-oxidation flux. Indeed, siRNA-mediated knockdown of both factors did not affect the ability of tBid to induce modifications on lipid metabolism, suggesting that this effect is mainly dependent on tBid alone. Interestingly, the only manipulations that effectively reduces the tBid-mediated mitochondrial dysfunction was transfection-enforced expression of Bcl-2. In fact, results of the current study have shown that the overexpression of Bcl-2 prevents the inhibition of β-oxidation in the hepatocytes treated with tBid. One mechanism whereby Bcl-2 may directly inhibit tBid-mediated effects is through the formation of Bcl-2/tBid complexes. These complexes were described in case of cells undergoing tumor necrosis factor-α-induced apoptosis.[43] Other studies suggest that Bcl-2 is able to decrease significantly the tBid insertion and/or integration in the mitochondrial membranes, a condition that strongly decreases the proapoptotic capacity of tBid.[16]

Alternatively, Bcl-2 may antagonize the action of tBid by interacting directly with CPT-1. Such interaction has been reported in various kinds of experimental models[15] and may inhibit the conformational changes in CPT-1 induced by tBid, thus preserving both enzymatic activity and mitochondrial β-oxidation flux.

Finally, the impaired CPT-1 activity could account for the increase of palmitoyl-CoA that we have demonstrated in tBid-treated cells. This increase can explain because mitochondria experience a progressive dysfunction in presence of tBid and without caspase activation. In fact, palmitoyl-CoA induces directly mitochondrial swelling and depolarization, thereby enhancing the irreversible mitochondrial alterations. Moreover, an accumulation of palmitoyl-CoA as a result of CPT-1 inhibition can influence the synthesis of ceramide, a molecule that play an important role in inducing programmed cell death. Thus, the alteration of mitochondrial fatty acid oxidation flux in apoptotic cells is a regulatory event of the cell death process rather than a by-product of apoptosis. This hypothesis suggests the existence of a system of lipid instructions that directly or indirectly, via the modulation of the killing activity of proapoptotic proteins, conditions the 'decision to die'.

Materials and Methods

Cell culture and chemicals

Primary human hepatocytes, culture reagents and media were purchased from Clonetics, (East Rutherford, NJ, USA). [U-^{14}C]Palmitate, [U-^{14}C] octanoate, [1-^{14}C]pyruvate and L-[*methyl*-^{14}C]carnitine were obtained from Amersham Pharmacia Biotech (Milan, Italy). Digitonin and zVAD-fmk were purchased from Sigma (Milan, Italy). Human recombinant tBid in PBS ($>$95% purity) was purchased from Alexis Biochemicals (Vinci, Italy) and used at a concentration of 20 ng/ml. In parallel, caspase-8-cleaved recombinant human Bid (R&D Systems, Milan, Italy) was used at a concentration of 30 ng/ml. The purification of tBid from R&D Systems did not employ detergent as depicted on the data sheet. No significant differences were seen between the two products on the ability to induce release of cytochrome *c* in isolated mitochondria or to inhibit CPT-1 activity in permeabilized cells. All the experiments showed in the paper were performed using the product from Alexis Biochemicals.

Analysis of $\Delta\Psi_m$ in permeabilized primary hepatocyte cells

Before permeabilization, hepatocytes were suspended in buffer A containing 220 mM mannitol, 68 mM sucrose, 10 mM Hepes (pH 7.4), 70 mM KCl, 1 mM EGTA, 1 mM phenyl methyl sulfonyl fluoride (PMSF) and 2 μM aprotinin. Different amounts of digitonin were added to cell preparations for choosing the quantity of molecule able to permeabilize selectively the plasma membrane without affecting mitochondria integrity. Citrate synthase and lactate dehydrogenase activities were measured by standard procedures to evaluate the integrity of mitochondria and the effectiveness of cell permeabilization process.[44,45]

For $\Delta\Psi_m$ analysis, permeabilized cells were incubated in the buffer A for 30 min at 37°C in the presence or absence of tBid (20 ng/ml), with or without cytochrome *c* (100 μM), and/ or zVAD-fmk (100 μM). Finally, the hepatocytes were incubated with 2 mM ADP, 2 mM DTT, 50 nM tetramethylrhodamine ethyl ester (TMRE) and 5 mM succinate and analyzed by flow cytometry on a FACScan (Becton Dickinson) measuring TMRE fluorescence in FL-2.

Isolation of mitochondria and Western blot analysis for cytochrome *c* release

Mitochondria were isolated as described in detail previously.[46] The isolation procedure was performed at 4°C. Briefly, hepatocytes were resuspended in buffer A, incubated for 30 min in the presence or absence of tBid, and disrupted using a Dounce with a tight fitting Teflon pestle. Mitochondrial pellet (P) and supernatant (S) fractions were obtained by spinning the mixture at $8000 \times g$ for 5 min. Hypotonically lysed mitochondria from untreated cells were used as positive controls; they were prepared as described previously.[47] Cytochrome *c* immunoblotting of the two fractions was performed with a commercially available anti-cytochrome *c* monoclonal antibody (Pharmingen, Milan, Italy). The blots were visualized by the ECL method (Amersham Pharmacia Biotech, Milan, Italy). To confirm that mitochondrial fraction was successfully separated from the cytosolic fraction, both fractions were probed with cytochrome c oxidase polypeptide IV (COX4) antibody (R&D Systems, Milan, Italy) by Western blot procedure.

Measurement of fatty acid oxidation and analysis of acylcarnitine intermediates in tBid treated-hepatocytes

Permeabilized hepatocytes were suspended in a buffer containing 110 mM KCl, 1 mM EGTA, 5 mM $MgCl_2$, 10 mM potassium phosphate, 1 mM ADP and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid with or without tBid for 30 min at 37°C in a shaking water bath (150 strokes/min). After this preincubation period, 5 mM ATP, 0.1 mM CoA, 1 mM L-carnitine, 100 μM cytochrome *c* and 50 μM [U-^{14}C]palmitate, complexed to fatty acid-free albumin in a 5 : 1 molar ratio (specific radioactivity 50 Ci/mol), were added. For the flux measurements, aliquots (50 μl) were quenched at timed intervals with an equal volume of glacial acetic acid. Unreacted substrate was removed by the addition of fatty-free albumin (0.1 ml of a 25%, wt/vol, water solution) followed by precipitation with 0.1 ml of 2 M perchloric acid. After centrifugation, acid-soluble radioactivity was measured in an aliquot of the supernatant by scintillation counting. For determination of intermediates, the remainder of the incubation mixture was quenched after 60 min by the addition of 100 ml of 1 M H_2SO_4, (see below).

For quantitation of $^{14}CO_2$ production, incubations were performed in glass vials, fitted with rubber caps, containing a 0.4 ml microcentrifuge tube inside a 1.5 ml microcentrifuge tube. After quenching with 100 μl of 2 M perchloric acid, 400 μl of hyamine hydroxide (40% in methanol) was added to the central microcentrifuge tube and left overnight to ensure complete trapping of $^{14}CO_2$. Total oxidation products were calculated as the sum of acid-soluble products and CO_2.

To evaluate $^{14}CO_2$ production by hepatocytes in the presence of substrates other than palmitate, [U-^{14}C]pyruvate or [U-^{14}C]octanoate (specific radioactivity 50 Ci/mol) were added to permeabilized cells at a final concentration of 0.1 mM. In some experiments, hepatocytes were incubated with etomoxir (100 μmol/l), rotenone (5 mg/l), antimycin A (5 mg/l) or sodium cyanide (1 mmol/l) indicated in each case. Etomoxir is the generic name for (+)-2-[6-(4-chlorophenoxy)hexyl]-oxiranecarboxylate, a compound able to inhibit specifically CPT-1 enzyme activity (Sigma-Aldrich, Milan, Italy). Total oxidation products were calculated as the sum of acid-soluble products and CO_2.

Acyl-CoA and acylcarnitines esters were extracted from the cell incubations and analyzed by radio-HPLC.[48] The identity of each acyl-CoA and acylcarnitine ester produced during the fibroblast incubations was determined by its relative retention time compared with those of standard compounds. Quantification was based on the integrated radioactive peaks after correction for recovery with appropriate internal standard.

Intracellular levels of malonyl-CoA were determined in neutralized perchloric acid cell extracts by a radioenzymatic method as described before.[49]

Measurement of CPT-1 in permeabilized cells

CPT-1 was measured by using digitonin-permeabilized cells as described by Sleboda *et al*.,[50] with minor modifications. A total of 10^6 primary human hepatocytes were plated in DMEM with 10% FBS in six-well plates in triplicate. Before CPT-1 measurement, the medium was removed, and cells were permeabilized and treated with tBid and zVAD-fmk as specified previously. Then, the hepatocytes were incubated with 700 μl of assay medium consisting of 50 mM imidazole, 70 mM KCl, 80 mM sucrose, 1 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 1 mM KCN, 1 mM ATP, 0.1% fatty acid-free BSA, 70 μM palmitoyl-CoA and 0.25 μCi of L-[*methyl*-^{14}C]carnitine, with or without etomoxir (100 μmol/l). After incubation for 5 min at 37°C, the reaction was stopped by the addition of 500 μl of ice-cold 4 M perchloric acid. Cells were then harvested and centrifuged at $13\,000 \times g$ for 5 min. The pellet was washed with 500 μl of ice-cold 2 mM perchloric acid and centrifuged again. The resulting pellet was resuspended in 800 μl of deionized H_2O and extracted with 400 μl of butanol. The butanol phase was counted by liquid scintillation. The assay was linear for up to 7 min at 37°C.

Quantitative analysis of cardiolipin in tBid-treated hepatocytes

Lipids were extracted from cell homogenates (10×10^6 cells/sample) according to Bligh and Dyer[51] Tetrastearoyl-cardiolipin (3.6 nmol) was added as an internal standard at the initial step of lipid extraction. At the end of procedure, the eluate was dried and redissolved in 0.1 ml of *n*-hexane-ethanol 1 : 1 (by volume). A measeure of 30 μl of this solution were separated by HPLC using a C18-Hypersil (5 mm) column (150×3.2 mm^2) as described previously.[52] A solvent gradient was run from acetonitrile–2-propanol 8 : 2 (by volume) to acetonitrile–2-propanol

5 : 5 in 30 min. For peak identification, fractions were collected and processed for measurement of the fatty acid profile.

siRNA-mediated knockdown of Bax and Bak

Hepatocytes were plated in six-well plates and transfected with Oligofectamine (Invitrogen) according to the manufacturer's protocol. siRNA against Bax and Bak were purchased from Dharmacon's SMARTpool selection. A negative control pool was included in all siRNA experiments. At 48 h after transfection, cells were split into fresh media for another 24 h before permeabilization. The expression of Bax and Bak was analyzed by Western blot using a primary anti-Bax (N-20) or anti-Bak (G-23) antibody (Santa Cruz Biotechnology, Milan, Italy) and a secondary HRP-conjugated anti-mouse IgG (Pierce, Milan, Italy). STAT-1 and ISG 15 antibodies were purchased by Delta Biolabs (CA, USA) and Novus Biologicals (CO, USA), respectively.

Hepatocyte transfection with Bcl-2

Hepatocytes were transfected with either Bcl-2 cDNA-containing neomycin-selectable pSFFVneo/Bcl-2 plasmid (HEP/Bcl-2 cells), or control neomycin-selectable pBK-CMV plasmid (HEP/neo cells) using Lipofectin (Life Technologies Inc., Gaithersburg, MD, USA) following the manufacturer's instructions. Several positive HEP/Bcl-2 and HEP/neo clones were selected and expanded. Cells were routinely maintained in a neomycin selection culture medium. The expression of Bcl-2 was confirmed by Western blot analysis using a primary anti-Bcl-2 antibody (clone Bcl-2/100 at 1 : 1000 dilution; Pharmingen, Milan, Italy) and a secondary HRP-conjugated anti-mouse IgG (1 : 5000 dilution; Pierce, Milan, Italy).

Evaluation of interaction of CPT-1 with tBid by immunoprecipitation

Immunoprecipitation of tBid/CPT-1 complexes was performed on hepatocytes or isolated mitochondria using a method described previously.[53] Hepatocytes were permeabilized and subjected or not to tBid treatment. Then, mitochondria were isolated and incubated with 10 mM bismaleimidohexane (BMH) crosslinker. The mitochondrial pellets were lysed in RIPA buffer. The extracts were directly used for immunoblot analysis of tBid, or subjected to immunoprecipitation with anti-CPT-1 antibody followed by detection of tBid in the precipitates by immunoblotting.

In another set of experiments, mitochondria were isolated from hepatocytes, incubated with 20 ng/ml tBID for 30 min at 30°C, treated with 10 mM BMH crosslinker and then lysed in RIPA buffer. Immunoprecipitation (with anti-CPT-1 antibody) and/or immunoblotting (with anti-tBid antibody) were performed as described before.

The composition of RIPA buffer was (in mM): 150 NaCl, 1 $MgCl_2$, 1 EGTA, 10 β-mercaptoethanol, 15 Tris-HCl, pH 7.4, containing 0.5% sodium deoxycholate, 0.2% SDS and 1% Triton X-100.

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