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Rapid report

Bcl-2 and tBid proteins counter-regulate mitochondrial potassium transport

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Abstract

The mechanism of cytochrome *c* release from mitochondria in apoptosis remains obscure, although it is known to be regulated by bcl-2 family proteins. Here we describe a set of novel apoptotic phenomena—stimulation of the mitochondrial potassium uptake preceding cytochrome *c* release and regulation of such potassium uptake by bcl-2 family proteins. As a result of increased potassium uptake, mitochondria undergo moderate swelling sufficient to release cytochrome *c*. Overexpression of bcl-2 protein prevented the mitochondrial potassium uptake as well as cytochrome *c* release in apoptosis. Bcl-2 was found to upregulate the mitochondrial potassium efflux mechanism—the K/H exchanger. Specific activation of the mitochondrial K-uniporter led to cytochrome *c* release, which was inhibited by bcl-2. tBid had an opposite effect—it stimulated mitochondrial potassium uptake resulting in cytochrome *c* release. The described counter-regulation of mitochondrial potassium transport by bcl-2 and Bid suggests a novel view of a mechanism of cytochrome *c* release from mitochondria in apoptosis.

Keywords: Apoptosis; Mitochondrion; Cytochrome *c*; Potassium transport; bcl-2; tBid

There are currently two major contending hypotheses on the mechanism of cytochrome *c* release from mitochondria in apoptosis [1]. One suggests that cytochrome *c* exits the mitochondrial intermembrane space via ruptures in the outer mitochondrial membrane during swelling, mediated by the mitochondrial permeability transition (MPT) [2]. The other advocates MPT-independent permeabilization of the outer mitochondrial membrane caused by pro-apoptotic bcl-2 family proteins, such as Bax [3]. It is not clear how pro-apoptotic bcl-2 family proteins induce permeabilization of the outer mitochondrial membrane and release of cytochrome *c* or how the anti-apoptotic protein, bcl-2, protects mitochondria and prevents the release of cytochrome *c*. In earlier work [4] we observed transient bcl-2-sensitive mitochondrial swelling, coincident with potassium accumulation in the organelles and preceding the MPT during apoptosis. The observed modification of mitochondrial potassium transport during apoptosis may be another mechanism involved in cytochrome *c* release in addition to the MPT and swelling-independent Bax-mediated release. This motivated further study on mitochondrial potassium transport in apoptosis and the effect of bcl-2 family proteins on it. Mitochondrial potassium transport, represented by uptake via the K-uniporter (KATP channel) and efflux via the K/H-exchanger, controls mitochondrial volume [5]. Net uptake of potassium leads to a volume increase, which may be sufficient to make the outer mitochondrial membrane permeable to cytochrome *c*.

As a model system, we chose the HL-60 cell line stably transfected (as described in Ref. [4]) with either *bcl-2* (HL-60/bcl-2) or with the empty vector (HL-60/neo) as a control. Apoptosis was induced with etoposide. In HL-60/neo cells, cytochrome *c* release from mitochondria started between 1.5 and 2 h after the beginning of treatment (Fig. 1A, top). This was followed by activation of caspase-3 (Fig. 1A, bottom). Overexpression of bcl-2 in HL-60/bcl-2 cells inhibited cytochrome *c* release as well as caspase-3 activation.

To evaluate the function of the mitochondrial K-uniporter during apoptosis, we rapidly isolated mitochondria at the indicated times after the induction and measured potassium influx via the uniporter. The assay revealed stimulation of

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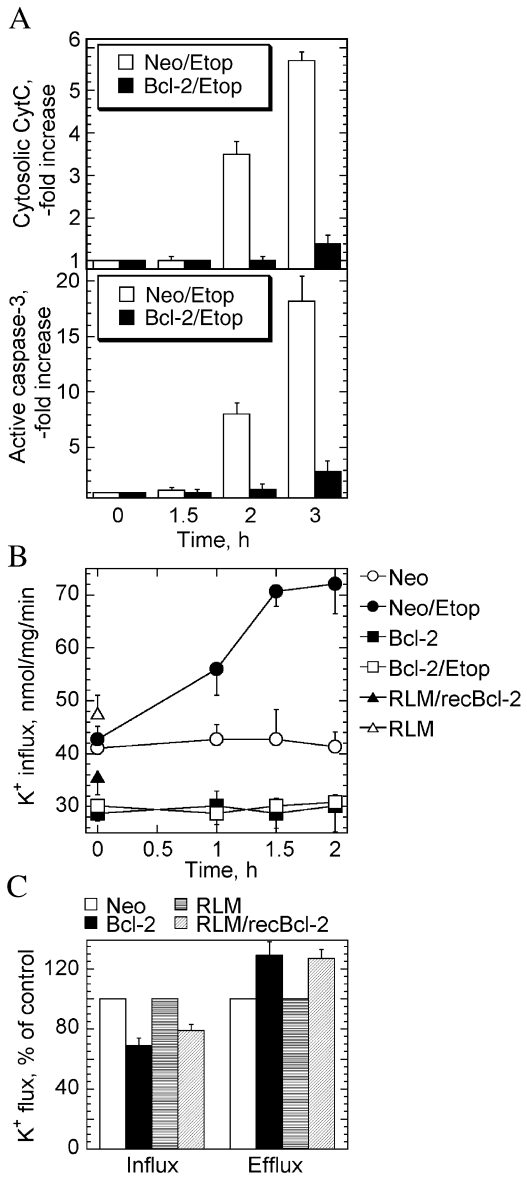

Fig. 1. Cytochrome *c* release and mitochondrial potassium transport during apoptosis in HL-60 cells. (A) Apoptosis was induced with etoposide and cytosolic fractions prepared at indicated times as described in [4]. Cytosolic cytochrome *c* (top panel) was measured by Western blotting and densitometry; active caspase-3 was measured by cleavage of Ac-DEVD-amc [6] (bottom panel). (B) K^+ influx into mitochondria isolated from etoposide-induced HL-60 cells at indicated times as in Ref. [4] or from intact rat liver as in Ref. [7]. K^+ influx calculated from the rate of swelling in K^+ acetate (KAc) [8], measured by light scattering. Recombinant Bcl-2 (recBcl-2) (R & D Systems), 1 μg/ml, was added to RLM where indicated. (C) Effect of bcl-2 on K^+ fluxes via the mitochondrial K-uniporter (Influx) and K/H-exchanger (Efflux). K^+ influx was measured as described above; K^+ efflux was assayed as in Ref. [9] by measuring light scattering changes caused by mitochondrial swelling due to net uptake of K^+ via a K/H-exchanger working in a reverse mode. Data are means ± S.D. ($n = 5$).

mitochondrial potassium influx in induced HL-60/neo cells, noticeable as early as at 1 h and reaching maximum at 1.5 h after the beginning of treatment, i.e. before the release of cytochrome *c* (Fig. 1B). The permeability of mitochondria to other ions, such as sodium, was also tested during apoptosis and found to be unchanged, suggesting that the effect was specific for potassium (Data not shown). The increased potassium influx into mitochondria may be a reason for the transient potassium accumulation and swelling of mitochondria documented in our earlier report [4].

Importantly, in both untreated and etoposide-treated HL-60/bcl-2 cells, the potassium influx remained unchanged and significantly lower than that in untreated HL-60/neo cells. The effect of bcl-2 was further studied on isolated rat liver mitochondria (RLM). The influx of potassium in control RLM was comparable to that in control HL-60/neo mitochondria. The addition of recombinant bcl-2 protein (recBcl-2) to the RLM led to the inhibition of potassium influx (Fig. 1B), further proving a limiting effect of bcl-2 protein on mitochondrial potassium uptake. We have therefore shown that both overexpression of bcl-2 in HL-60 cells and addition of recBcl-2 to RLM led to the decrease in net potassium influx into mitochondria (Fig. 1C). However, the observed reduction of the net potassium influx could be due to the increased efflux of potassium via the K/H-exchanger. To test this hypothesis, we measured the potassium flux via the mitochondrial K/H-exchanger in both mitochondria from HL-60 cells and RLM in the presence or absence of bcl-2. The assay revealed that overexpression of bcl-2 led to the increased potassium flux via the mitochondrial K/H-exchanger in the HL-60/bcl-2 cells compared to the HL-60/neo cells, and that in RLM addition of recBcl-2 also resulted in increased potassium flux via the K/H-exchanger (Fig. 1C). These data indicate that bcl-2 upregulates the mitochondrial K/H-exchanger, thereby increasing potassium efflux and facilitating the removal of excess potassium from mitochondria, resulting in decreased net uptake of potassium by mitochondria.

Mitochondrial potassium uptake via the K-uniporter results in a slight depolarization of mitochondria [10], incomparable to a drastic MPT-induced depolarization [11]. We, therefore, measured the mitochondrial membrane potential ($\Delta\Psi m$) in cells treated with etoposide. The assay revealed only an insignificant decrease in $\Delta\Psi m$ in the HL-60/neo cells, consistent with potassium uptake at a time preceding cytochrome *c* release (Fig. 2A). We also measured oxygen consumption of mitochondria isolated at the indicated times from HL-60/neo and HL-60/bcl-2 cells. Oxygen consumption was found to be higher in mitochondria from cells treated with etoposide for 1.5 h (Fig. 2B), i.e. at the time when the mitochondrial potassium influx reached maximum. Influx of potassium into mitochondria inevitably results in some stimulation of respiration [10], therefore the oxygen consumption data are consistent with potassium influx data. It is of importance that the respiration was equally stimulated in HL-60/bcl-2 cells, although the net potassium influx was lower compared to that in HL-60/neo cells. This may be an evidence that in induced HL-60/bcl-2 cells, the potassium flux via the mitochondrial K-uniporter was increased, leading to the observed stimulation of respiration, but the net

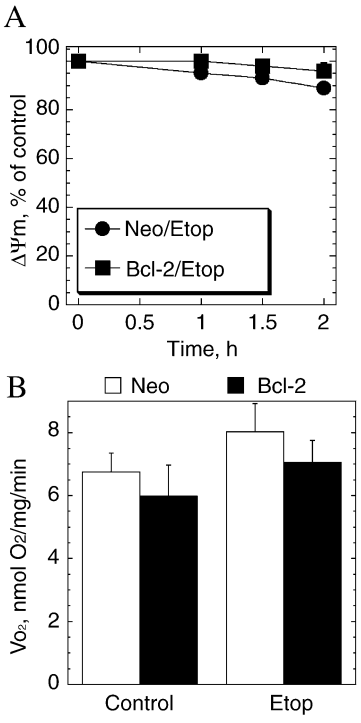

Fig. 2. Mitochondrial membrane potential (ΔΨm) and oxygen consumption in apoptosis. (A) ΔΨm was measured by monitoring DNP-sensitive TPP^+ uptake [12] into mitochondria in digitonin-permeabilized cells [13]. TPP^+ was measured with a pre-calibrated TPP^+-sensitive electrode. (B) Oxygen consumption in mitochondria isolated from HL-60 cells was measured with a Clark oxygen electrode [14]. Data are means ± S.D. ($n=3$).

potassium influx into mitochondria remained low due to the upregulation of potassium efflux by bcl-2.

To find out if a specific stimulation of mitochondrial potassium uptake via the K-uniporter is sufficient to release cytochrome *c*, we incubated cells with diazoxide. At low concentration (30 μM), diazoxide only opens the mitochondrial K-uniporter and not the plasma membrane K-channel [10]. We found that diazoxide-induced stimulation of mitochondrial potassium uptake results in cytochrome *c* release from mitochondria (Fig. 3A, top), which is inhibitable by a specific inhibitor of the mitochondrial K-uniporter 5-hydroxydecanoate (5-HD) as well as by overexpression of bcl-2 as in HL-60/bcl-2 cells. The released cytochrome *c* was enough to activate caspase-3, an event also prevented by both 5-HD and bcl-2 overexpression (Fig. 3A, bottom). In isolated RLM, stimulation of mitochondrial potassium uptake resulted in swelling, which was significantly slower and less pronounced than MPT-induced swelling (Fig. 3B), but was nevertheless sufficient to release cytochrome *c* (Fig. 3C). Both potassium-induced swelling and cytochrome *c* release in RLM were inhibited by addition of recBcl-2. The effect of bcl-2 was similar to the effect of ATP—a known inhibitor of the mitochondrial K-uniporter, also-called the KATP channel because of its sensitivity to ATP [10]. These data confirm that stimulation of mitochondrial potassium uptake via the K-uniporter both in situ and in vitro is sufficient to release cytochrome *c* from mitochondria, an event inhibited by bcl-2.

We have therefore shown that bcl-2 protein limits potassium uptake into mitochondria most likely by upregulating the mitochondrial potassium efflux mechanism—K/H-exchanger. However, it was not clear which apoptotic factor might trigger observed bcl-2 sensitive potassium uptake via

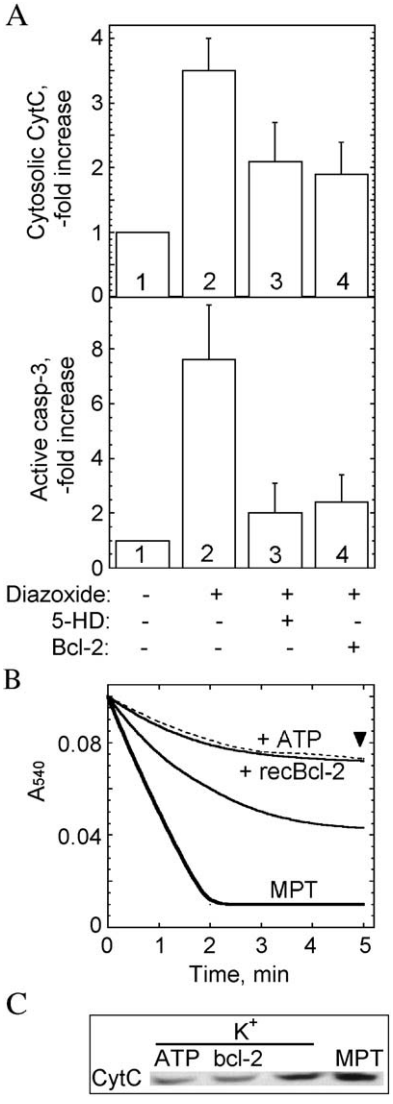

Fig. 3. Induction of mitochondrial potassium influx via a K-uniporter in situ and in vitro results in cytochrome *c* release sensitive to bcl-2. (A) HL-60/neo (columns 1–3) and HL-60/bcl-2 (column 4) cells were incubated for 3 h with 30 μM diazoxide, where indicated, in the presence or absence of 300 μM 5-HD. Cytosolic cytochrome *c* and active caspase-3 were measured as described in Fig. 1. Data are means ± S.D. ($n=3$). (B) Swelling of RLM was determined by decrease in light scattering. Conditions are the same as in Fig. 1B. RLM swell in KAc (unlabeled curve). recBcl-2 (+recBcl-2), 1 μg/ml, inhibits potassium-induced swelling similarly to 200 μM ATP (+ATP). MPT-mediated swelling (MPT) is much faster and more pronounced. MPT induced with 0.5 mM $CaCl_2$. Arrowhead indicates time of withdrawing samples for cytochrome *c* assay. (C) Samples of RLM after 5 min of either K^+ uptake or the MPT were taken as indicated in B; mitochondria were pelleted and the supernatant analyzed by Western blotting for cytochrome *c*.

the mitochondrial K-uniporter during apoptosis. In searching for such a trigger, we tested the effect of the pro-apoptotic bcl-2 family proteins Bax and Bid on the mitochondrial K-uniporter as well as on the mitochondrial K/H-exchanger. Bax and Bid are commonly known factors that translocate into mitochondria and induce cytochrome *c* release [3]. It is known that a truncation of the N-terminal of Bid by caspase-8 [15] or of the N-terminal of Bax by calpain [16] increases their ability to translocate into mitochondria and release cytochrome *c*. For that reason, we purchased N-terminal truncated Bid, tBid, and produced a recombinant N-terminal truncated Bax, tBax, and studied their effect on isolated mitochondria (RLM). The addition of tBax to RLM resulted in release of cytochrome *c* (Fig. 4A) without any effect on the mitochondrial potassium uptake, efflux or $\Delta\Psi m$ (Data not shown). This indicates that Bax permeabilizes the outer mitochondrial membrane to cytochrome *c* via a potassium- and swelling-independent pathway, confirming the previous results from other groups [17]. In the presence of bcl-2, tBax-induced cytochrome *c* release was only diminished but not completely stopped, possibly due to the fact that we used the highly active form of Bax—tBax. Meanwhile, the addition of tBid to isolated RLM drastically increased the mitochondrial potassium uptake via the K-uniporter in a bcl-2-sensitive way (Fig. 4B), resulting in a bcl-2-sensitive release of cytochrome *c* (Fig. 4C). The mitochondrial K/H-exchanger was unaffected. The effect of tBid was further proved by using a different assay of mitochondrial K^+ uptake. We monitored matrix potassium with a K^+-sensitive fluorescent probe PBFI (Fig. 4D). Respiring control RLM resuspended in a KCl-based buffer took up K^+ (unlabeled curve). Addition of valimomycin as a positive control resulted in drastic increase in matrix K^+. The K-uniporter inhibitor, glibenclamide, stopped K^+ uptake in RLM. The same was observed in the presence of recBcl-2. tBid activated K^+ uptake, and the combination of tBid and Bcl-2 resulted in K^+ uptake identical to control (not shown). These findings indicate that tBid may be the stimulus that triggers the observed potassium uptake into mitochondria during apoptosis.

Our results show that bcl-2 family proteins regulate mitochondrial potassium transport. Anti-apoptotic bcl-2 upregulates the potassium efflux mechanism, the K/H-exchanger, and prevents potassium uptake into mitochondria, while pro-apoptotic Bid stimulates potassium influx into mitochondria via the K-uniporter. The stimulation of mitochondrial potassium uptake leads to the mitochondrial swelling which is less pronounced than the MPT-mediated swelling and not accompanied by a significant depolarization, but can be, however, sufficient to release cytochrome *c*. We have found that the activation of the mitochondrial K-uniporter occurs early in etoposide-induced apoptosis in HL-60 cells and precedes the release of cytochrome *c* from mitochondria. Both events, potassium uptake and cytochrome *c* release, are prevented by bcl-2 overexpression. The specific activation of mitochondrial potassium uptake via the K-uniporter results in cytochrome *c* release, which

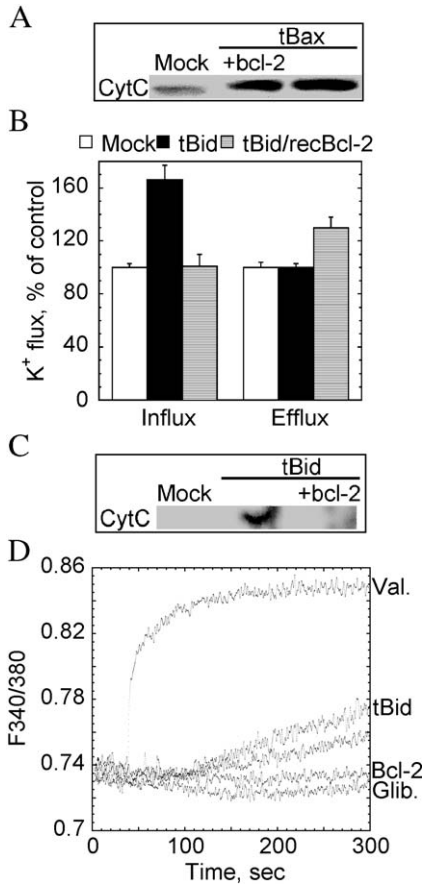

Fig. 4. Effect of recombinant tBax and tBid proteins on mitochondrial K^+ uptake and cytochrome *c* release. (A) Cytochrome *c* release induced by 1 μg/ml tBax. Recombinant tBax (Δ1–38 aa) was produced using MBP fusion system [18]. MBP tag was then cleaved with 3C protease, yielding pure tBax. Random sequence was expressed as a "mock" protein. (B) K^+ influx and efflux in RLM were measured as in Fig. 1 in the presence of 1 μg/ml recombinant tBid (R & D Systems) ± 1 μg/ml recBcl-2 or mock protein. Data are means ± S.D. ($n=3$). (C) Supernatants from RLM undergoing K^+ uptake in the presence of tBid ± recBcl-2 or of "mock" protein for 10 min were subjected to Western blotting for cytochrome *c*. (D) Matrix K^+ was measured using PBFI. Mitochondria were loaded with PBFI-AM for 20 min at r.t., washed and resuspended in KCl based buffer [14] with no further additions (unlabeled curve); 1 μM Valinomycin (Val.), 1 μg/ml tBid, 1 μg/ml Bcl-2 or 10 μM Glibenclamide (Glib.). $F_{340/380}$ was measured in a spectrofluorimeter. Data are representatives of three independent trials.

again is inhibitable by bcl-2. Bax did not affect the mitochondrial K-uniporter and released cytochrome *c* without any noticeable potassium uptake or depolarization.

The data presented here suggest a novel explanation for earlier observations of cytochrome *c* release from mitochondria in apoptosis and its regulation by bcl-2 family proteins. The existing controversy over the mechanism of cytochrome *c* release arises from insufficient evidence that either the MPT-mediated swelling or swelling-independent Bax/Bid-mediated channel plays a major role in permeabilization of the outer mitochondrial membrane to cytochrome *c*. Although mitochondrial swelling has been frequently observed in apoptosis [19], depolarization—a hallmark of

the MPT—is not always obvious [20], neither is the effect of a specific MPT inhibitor, cyclosporin a [4]. Moreover, Bax and Bid have been shown to release cytochrome *c* from isolated mitochondria independently of the MPT [21]. Scorrano et al. [22] showed that Bid causes MPT-independent rearranging of mitochondrial cristae, opening of intracristae spaces and blebbing of mitochondria resulting in rupture of the outer membrane. Such conformational changes are impossible without changes in matrix volume, the major regulator of which is the osmotically active potassium transport. Our results may clarify the mechanism of action of Bid. Observed stimulation of the mitochondrial potassium uptake via the K-uniporter induced by tBid may change the conformation of cristae and result in a moderate MPT-independent volume increase and blebbing.

We did not detect any effect of Bax on the mitochondrial potassium uptake, suggesting a different, potassium-independent mechanism of Bax action. Bax may form channels that facilitate translocation of cytochrome *c* across the outer mitochondrial membrane in the absence of swelling or conformational changes of mitochondria induced by either potassium uptake or the MPT.

Our study suggests novel aspects of apoptotic signaling, the involvement of the mitochondrial potassium transport systems in cytochrome *c* release, and counter-regulation of the potassium transporters by anti-apoptotic bcl-2 protein and pro-apoptotic Bid protein.

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