Differential Effects of Bcl-2 on Cell Death Triggered under ATP-Depleting Conditions

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The intracellular ATP concentration decides on the onset of either apoptosis or necrosis in Jurkat cells exposed to death stimuli. Bcl-2 can block apoptotic demise, which occurs preferably under conditions of high cellular ATP levels. Here, we investigated the effects of Bcl-2 on the necrotic type of cell demise that prevails under conditions of energy loss. ATP levels were modulated by using mitochondrial inhibitors, such as rotenone or *S*-nitrosoglutathione, in medium either lacking glucose or supplemented with glucose to stimulate glycolytic ATP generation. Under conditions of ATP depletion, staurosporine (STS) induced >90% necrosis in vector control-transfected cells, whereas *bcl-2*-transfected cells were protected. Thus, the antiapoptotic protein Bcl-2 can reduce the overall amount of cell death in ATP-depleted cells regardless whether it occurs by apoptosis or necrosis. Cytochrome *c* release, normally preceding STS-induced necrosis, was also inhibited by Bcl-2. However, Bcl-2 did not prevent an initial STS-induced drop of the mitochondrial membrane potential ($\Delta\Psi_m$). Therefore, the mechanisms whereby Bcl-2 prevents cell death and favors retention of cytochrome *c* in the mitochondria require neither the maintenance of mitochondrial $\Delta\Psi$ nor the maintenance of normal ATP levels. © 2001 Academic Press

Key Words: apoptosis; necrosis; NO; ATP; Bcl-2; cytochrome *c;* mitochondria.

INTRODUCTION

Tissue damage and its pathological consequences depend on the amount (overall cell loss) and the mode (apoptosis or necrosis) of cell death. One important factor deciding the mode of death is the intracellular ATP[2] level [1, 2]. Depletion of adenine nucleotide triphosphates below a certain threshold blocks the execution of apoptosis, while it does not affect ATP-independent pathways that lead to necrosis [1–3]. Conditions that cause such an ATP depletion *in vivo* comprise a variety of ischemic or toxic insults or oxidative stress.

Overexpression of the antiapoptotic protein Bcl-2 reduces the overall amount of cell loss in many models of apoptosis [4–7]. Several mechanisms have been implicated to explain protective effects of Bcl-2. It can prevent the loss of the mitochondrial membrane potential, the release of mitochondrial cytochrome *c* and AIF, and the subsequent caspase activation and chromatin degradation in many systems [8–11]. In addition, Bcl-2 may have other targets in the nucleus and the endoplasmic reticulum that may be relevant for its antiapoptotic effects [12, 13]. Despite its broad range of action, some forms of apoptosis are not prevented by *bcl-2* overexpression [14–20] and in some cases *bcl-2* overexpression enhances cell death [21]. The decision on whether or not apoptosis pathways are controlled by Bcl-2 can depend on the cell type (e.g., CD95 ligation can trigger in some cells Bcl-2-sensitive and in others Bcl-2-insensitive death pathways) [22]. Moreover, the existence of multiple death pathways within one cell type may explain Bcl-2-sensitive or insensitive death cascades [15, 23]. Finally, pharmacological intervention may also reverse Bcl-2 protection [24].

In contrast to the extensive research on the antiapoptotic effects of Bcl-2, the data on the effects of Bcl-2 on necrotic cell death are limited. In particular it has remained unclear, whether Bcl-2 can protect cells that have initially been triggered to undergo apoptosis, but are then executing a different type of cell death because of ATP depletion. This question has gained considerable importance, since strategies to regulate Bcl-2 levels have been discussed as clinical approaches to prevent excessive cell death in situations that in-

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[2] Abbreviations used: ATP, adenosine triphosphate; *bcl-2* cells, *bcl-2*-transfected cells; DEVD-afc, DEVD–aminotrifluoromethylcoumarine; GSNO, *S*-nitrosoglutathione; neo cells, vector-transfected cells; NO, nitric oxide; PNG, glucose-free medium supplemented with pyruvate; STS, staurosporine; TMRE, tetramethylrhodamine ethylester; $\Delta\Psi_m$, mitochondrial membrane potential.

volve ATP depletion, e.g., in stroke and organ failure. Previous work has investigated the effects of Bcl-2 in models of necrosis elicited by oxidative stress or hypoxia. Bcl-2 delayed cell death or reduced the amount of dying cells in models of mild transient stress [25–28]. In these systems, several death pathways are probably activated, some of which may kill cells independently from the loss or retention of adequate ATP levels.

In this study, we used a different approach to investigate the potential protective effect of Bcl-2 on necrosis occurring under poor metabolic conditions. ATP levels of Jurkat cells were reduced in a controlled manner by inhibition of oxidative phosphorylation using rotenone or nitric oxide donors in a glucose-free environment. Cells were then exposed to death stimuli. As control, ATP was repleted in the presence of the inhibitors by supplying the cells with glucose. Under these conditions, a variety of substances known to induce apoptosis in cells with normal ATP levels caused swelling and necrotic lysis in ATP-depleted cells within a time frame in which energy failure per se did not have lethal effects [1, 3, 19, 29]. In this model, we examined the effects of Bcl-2 on cell death, loss of mitochondrial membrane potential, and cytochrome c release.

MATERIALS AND METHODS

Materials. SYTOX and Hoechst 33342 were obtained from Molecular Probes (Eugene, OR). *S*-Nitrosoglutathione (GSNO) was synthesized and quantitated as described previously [30]. The caspase substrate DEVD–aminotrifluoromethylcoumarine (–afc) was obtained from Biomol (Hamburg, Germany). All other reagents not further specified were from Sigma (Deisenhofen, Germany), Merck (Darmstadt, Germany), or Riedel-de Haen (Seelze, Germany).

Cell clones and culture. Jurkat T cell clones stably transfected with the human *bcl-2* gene (*bcl-2*) or control vector (*neo*) were kindly provided by S. Korsmeyer (Dana Farber Cancer Institute, Boston, MA) and cultured exactly as described [31]. Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. Before experiments, cells were washed and resuspended (4×10^6 cells/ml) in serum- and glucose-free RPMI 1640 medium containing 2 mM pyruvate (PNG medium) to allow oxidative phosphorylation. After adaptation to this medium for 45 min, cells were exposed to GSNO (0 or 0.4 mM) or to rotenone (0 or 0.2 μM). After an additional 45 min, cells were challenged with 1 μM staurosporine (STS). Where indicated, the medium was supplemented with glucose (10 mM) in order to restore glycolytic ATP synthesis.

Viability assays. To distinguish normal, apoptotic, and necrotic cells, cultures were stained with a combination of the fluorescent chromatin dyes Hoechst 33342 (500 ng/ml, membrane permeant, stains all nuclei) and SYTOX (500 μM, membrane impermeant, stains nuclei of lysed cells) and viewed directly 5–10 min after addition of the dyes as described previously [1]. Using a Leica DM-IRB fluorescence microscope and lenses providing 400× final magnification, cells with condensed or fragmented nuclei were scored as apoptotic; lysed cells with noncondensed nuclei were scored as necrotic. For each data point >200 cells were scored in at least three different microscopic fields.

Determination of cytochrome c release. To determine cytochrome c release from mitochondria, cytosolic fractions were isolated after selective lysis of the plasma membrane with digitonin as described previously [3, 29]. Briefly, 4×10^6 cells were resuspended in 250 μl phosphate-buffered saline and lysed by adding 250 μl of a digitonin (100 μg/ml)/sucrose (500 mM) solution under vigorous vortexing, and further incubation for 30 s. Cytosolic fractions were quickly isolated and removed from organelles and cell debris by centrifugation at 14,000g for 60 s at 4°C. Aliquots for caspase activity determinations were withdrawn at this step. Protein in the remaining fraction was precipitated with 5% trichloric acid. As reference for total cytochrome c content of the cells, organelle-comprising fractions of untreated cells were resuspended in the digitonin solution and precipitated in parallel. Protein was dissolved in sample buffer (6 M urea, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 60 mM Tris, pH 6.8) and equal amounts (corresponding to 10^6 cells) were loaded, separated by SDS–polyacrylamide gel electrophoresis, and subsequently transferred to nitrocellulose membranes (Hybond-ECL, Amersham-Bucher Corp., Braunschweig, Germany). Protein content was routinely controlled by staining of the membranes with Ponceau Red. The membranes were blocked and immunoblotted with the primary antibodies against cytochrome c (7H8.2C12, PharMingen, Hamburg, Germany). Reactions were detected with a polyclonal IgG anti-mouse (1:1000, PharMingen), conjugated to horseradish peroxidase followed by enhanced chemiluminescence detection (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany).

Western blot for Bcl-2. For Bcl-2 analysis, cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EGTA), which was supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM iodoacetate, 1 mM iodoacetamide, 40 μM leupeptin, 10 μg/ml antipain, 5 μg/ml pepstatin). To detect the level of Bcl-2 in overexpressing cells, 20 μg protein/lane were separated on a 15% SDS–polyacrylamide gel. For endogenous Bcl-2 levels in vector-transfected cells, 50 μg protein/lane was loaded onto a 4–20% glycine gel (Novex, Frankfurt/Main, Germany). For direct comparison, 10 μg protein/lane of *bcl-2*-transfected cells was loaded on one lane of the same gel. Bcl-2 was demonstrated by immunoblotting with the antibodies against Bcl-2 (N-19, Santa Cruz Biotechnology, Santa Cruz, CA) followed by polyclonal IgG anti-rabbit (Sigma) and subsequent enhanced chemiluminescence detection.

ATP measurement and caspase activity. For ATP determination, 10^5 cells were lysed with ATP-releasing reagent (Sigma). ATP concentrations in lysates were determined luminometrically as described previously using a luciferase assay (Boehringer Mannheim, Mannheim, Germany) [1].

Cytosolic caspase activity was determined by measuring the cleavage of the fluorogenic substrate DEVD-afc exactly as described before [32]. Cytosolic fractions were obtained as described for cytochrome c determination and supplemented with EDTA (1 mM) and the serine-protease inhibitor AEBSF (1 mM). The fluorometric assay was performed in microtiter plates with sample volumes corresponding to 8×10^4 cells.

Mitochondrial membrane potential. The mitochondrial membrane potential $\Delta\Psi_m$ was measured by using the potential sensitive dye tetramethylrhodamine ethylester (TMRE) (Molecular Probes). Cells were loaded with 5 nM TMRE (a concentration which did not cause quenching of the fluorescence signal upon strong accumulation in mitochondria). Digital images of the cells were taken with a Leica DM IRB microscope coupled to a Dage-72 CCD camera (Dage-MTI, Michigan City, IN) and an image analysis system (Imaging Inc., St. Catherines, Ontario, Canada). Controls and precautions for ensuring reproducible conditions for lighting, loading, and image acquisition were taken as described before [19, 33, 34].

A

B

C
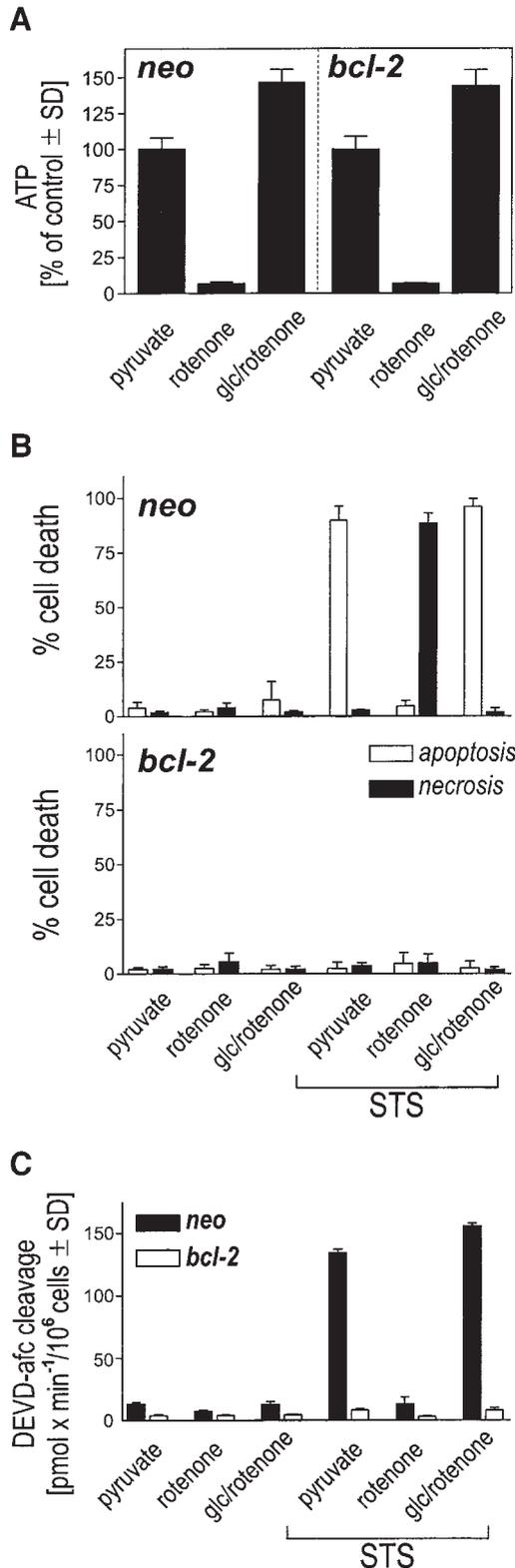
FIG. 1. Effect of Bcl-2 on STS-induced necrosis in rotenone-treated cells. Jurkat *neo* and *bcl-2* cells were preincubated for 45 min in PNG medium alone (*pyruvate*) or in the presence of 0.2 μM rotenone. Then, cells were exposed to 1 μM STS or solvent. Where

RESULTS

Delay of STS-Induced Death (Necrosis) by Bcl-2 in Jurkat Cells Depleted of ATP by Rotenone

Cellular energy levels of Jurkat cells were modulated by incubating control vector-transfected (*neo*) or *bcl-2*-overexpressing (*bcl-2*) clones in different media. As control condition, we used glucose-free medium enriched in pyruvate (termed PNG medium). Under these conditions, cells retained high ATP concentrations due to mitochondrial ATP synthesis (Fig. 1A). Addition of rotenone blocked oxidative phosphorylation and depleted ATP to less than 10% of control values within 45 min, both in *neo* and in *bcl-2*-transfected cells. When the medium of rotenone-treated cells was supplemented with glucose, ATP levels were instead retained. Rotenone-induced ATP depletion was tolerated by Jurkat cells for up to 10 h without resulting in significant cell death. After this time cells started lysing necrotically and Bcl-2 had a minor delaying effect that varied in individual experiments. After 15 h, more than 90% of both *bcl-2* and *neo* cells displayed necrotic features (not shown). Since Bcl-2 barely affected the slow cell death due to ATP depletion alone, we examined whether Bcl-2 would protect cells from a proapoptotic stimulus applied during the time frame when ATP depletion alone was not detrimental. *Neo* cells depleted of ATP by rotenone and challenged with STS lysed necrotically after 6–7 h (Fig. 1B). *Bcl-2*-transfected cells were instead protected and did not show any morphological features of necrosis for ~10 h.

Bcl-2 Protects from STS-Induced Necrosis in Cells Depleted of ATP by GSNO

Since Jurkat cells tolerated ATP depletion by the mitochondrial poison rotenone only for about 10 h, we decided to use a reversible and physiological mitochondrial inhibitor nitric oxide (NO). The NO donor GSNO has been shown to effectively lower ATP levels in Jurkat cells [3, 34]. After 45 min of treatment with GSNO, ATP levels in Jurkat cells had decreased below 35% of control values and remained low throughout the exper-

indicated, medium was supplemented with 10 mM glucose (*glc*). A, cellular ATP content was determined after the preincubation time. Data are given as percentages of control in PNG medium $\pm$ SD (controls were *neo,* 1.07 $\pm$ 0.02 nmol/10^6 cells; *bcl-2,* 1.14 $\pm$ 0.04 nmol/10^6 cells). B, STS-induced apoptosis (white bars) and necrosis (black bars) were quantitated by Hoechst 33342/SYTOX staining after 7 h of treatment with or without STS in *neo* cells and *bcl-2* cells. Data are means from triplicate determinations and are given as percentages of control in PNG-medium. C, caspase activity was determined after 4 h of STS treatment as DEVD-afc cleavage activity. Data are means $\pm$ SD from triplicate determinations.
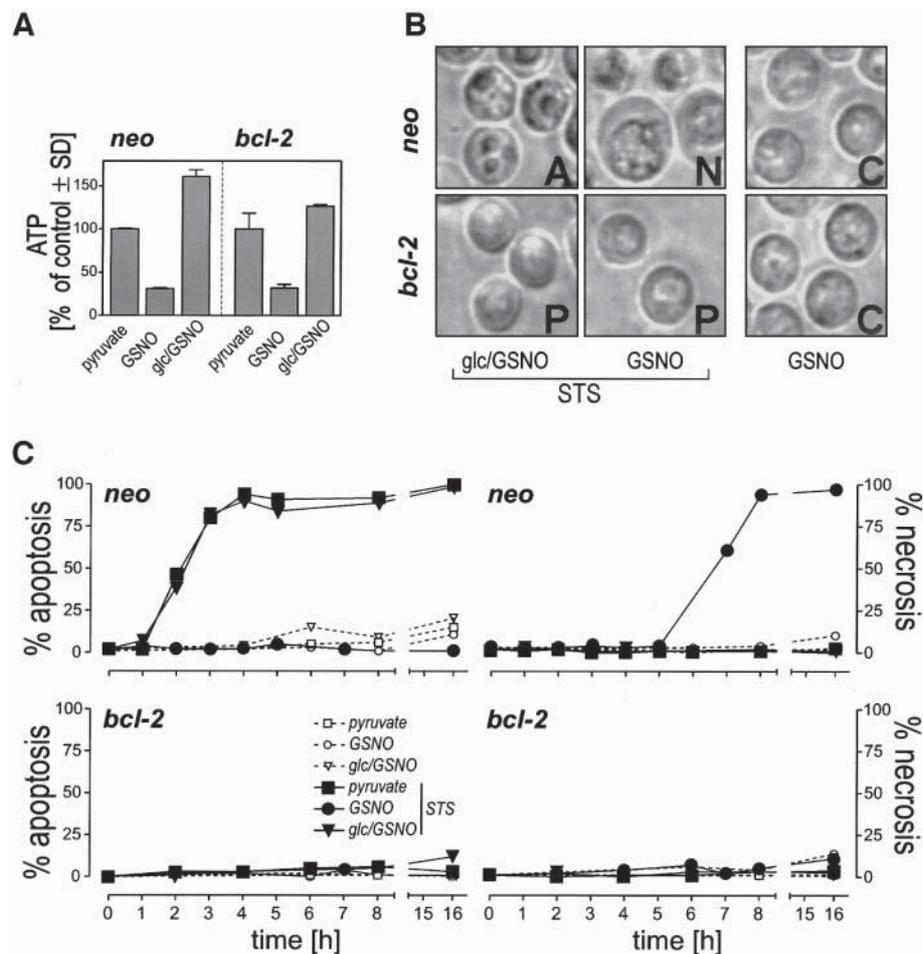
FIG. 2. Bcl-2 protects from STS-induced necrosis in cells depleted of ATP by GSNO. Jurkat *neo* and *bcl-2* cells were preincubated for 45 min in PNG medium alone (*pyruvate*) or supplemented with 0.4 mM GSNO or GSNO plus glucose (*glc/GSNO*). Cells were stimulated with 1 μM STS or solvent. A, the ATP content was determined at the time of STS addition. Data are given as percentages of control in PNG medium. B, phase contrast images of *neo* and *bcl-2* cells after 7 h of treatment; images show control (C), apoptotic (A), necrotic (N), and protected (P) cells. C, at the times indicated, apoptosis and necrosis were quantitated by Hoechst 33342/SYTOX staining. Data are means from three independent experiments.

imental period (Fig. 2A). This decrease was not affected by Bcl-2, indicating that Bcl-2 neither interfered with the inhibition of oxidative phosphorylation nor scavenged NO. This treatment did not affect viability of both *neo* and *bcl-2* cells for ≥16 h (Fig. 2C). Addition of glucose again restored ATP levels (Fig. 2A). ATP depletion by GSNO blocked STS-induced apoptosis, and instead cells swelled and lysed after 6–7 h of STS treatment (Figs. 2B and 2C). In *bcl-2* cells treated with STS, swelling and cell lysis were blocked for at least 16 h.

Structural and Functional Integrity of Bcl-2 under Conditions of ATP Depletion or NO Exposure

ATP depletion or STS treatment did not alter Bcl-2 levels in *bcl-2*-transfected cells (Fig. 3B). Also endog-

enous levels of Bcl-2 in *neo* cells did not show any detectable changes in either apoptosis or necrosis.

As an additional indication that Bcl-2 was fully functional in the presence of either GSNO or rotenone, we tested whether *bcl-2* would inhibit STS-induced caspase activity in the presence of these mitochondrial inhibitors. In PNG medium, *bcl-2* expression reduced caspase activation elicited by STS by >95% in accordance with the powerful antiapoptotic function of the oncogene (Figs. 1C and 3A). Caspases were not activated at any time in ATP-depleted *neo* or *bcl-2* cells before necrosis occurred. However, this was not due to a direct inhibition by NO or rotenone, since full caspase activation was observed in *neo* cells grown in glucose-containing medium (i.e., with high ATP levels) in the presence
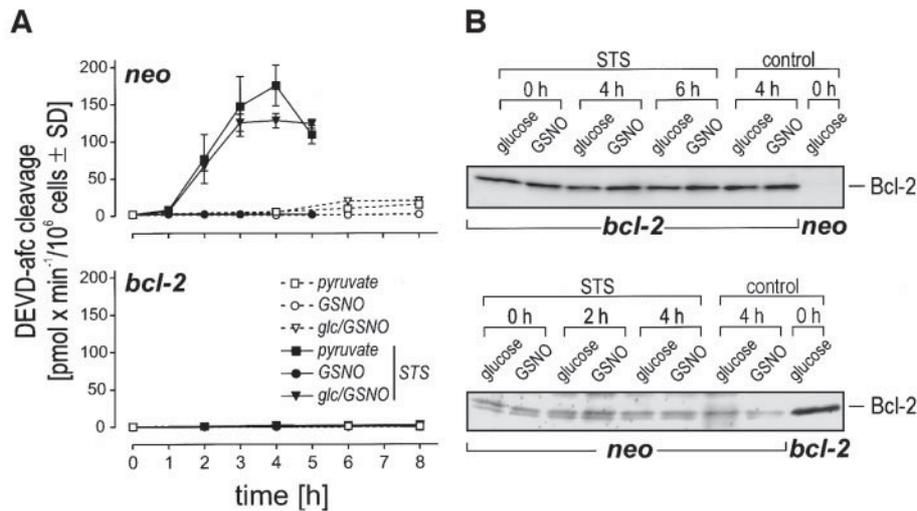
FIG. 3. Caspase activity and Bcl-2 levels in ATP depleted cells. A, Jurkat *neo* and *bcl-2* cells were incubated in PNG medium alone (pyruvate) or supplemented with 0.4 mM GSNO or GSNO plus glucose (glc/GSNO). Cells were stimulated with 1 μM STS or solvent. At the times indicated, caspase activity was determined as DEVD-afc cleavage. Data are means $\pm$ SD from triplicate determinations. B, Bcl-2 levels were determined by Western blot in stimulated and control cells in PNG medium supplemented with glucose or GSNO. To detect Bcl-2 levels in *bcl-2*-transfected cells, 20 μg protein/lane was loaded. For detection of endogenous Bcl-2 levels in *neo* cells, 50 μg protein/lane was loaded. For direct comparison, 10 μg protein/lane of *bcl-2*-transfected cells was loaded on one lane of the same gel. The additional lower band observed in *neo* cells was possibly due to protein overloading and did not correlate with treatment.

of either rotenone or GSNO. This activation was also entirely blocked by Bcl-2 in the presence of the mitochondrial inhibitors.

Bcl-2 Delays STS-Induced Loss of $\Delta\Psi_m$ and Prevents Cytochrome c Release in Rotenone-Treated Cells

The localization of cytochrome *c,* as well as the mitochondrial membrane potential ($\Delta\Psi_m$), were monitored in rotenone-treated cells. As control, both parameters were also examined in cells with high ATP levels: Apoptotic cytochrome *c* release started after 1 h of STS treatment and after 2–3 h the major fraction of cytochrome *c* was found in the cytosol (Figs. 4B and 5B). After 3–4 h of STS treatment, the majority of cells with initially high ATP levels had lost TMRE staining (Fig. 5A), and at this time, $\geq$90% of the cells showed apoptotic nuclei (Figs. 1A and 2A). According to its described antiapoptotic properties, Bcl-2 completely protected from the apoptotic loss of $\Delta\Psi_m$ and from cytochrome *c* release (Figs. 4B, 5A, and 5B).

When ATP-depleted cells were challenged by STS, cytochrome *c* release started after about 1.5 h and was maximal after 3–4 h. This release was inhibited by Bcl-2 (Fig. 4B). Only 6–8 h after the challenge, low amounts of cytosolic cytochrome *c* were detected in some experiments when ATP was depleted with rotenone (Fig. 4B). Such a late release was also observed in unstimulated ATP-depleted cells and preceded subsequent cell lysis (not shown).

Loss of $\Delta\Psi_m$ paralleled the cytochrome *c* release in *neo* cells: After 1.5 h, the majority of the Jurkat cell population had lost TMRE staining (Fig. 4A). Notably, Bcl-2 was not able to inhibit this loss but only delayed it by about 1.5 h. The failure of Bcl-2 in maintaining $\Delta\Psi_m$ was also evident in nonchallenged Jurkat cells depleted of ATP by rotenone: after 6 h all TMRE staining was lost independently of the Bcl-2 status (not shown). The failure of Bcl-2 to maintain $\Delta\Psi_m$ may explain its inability to provide permanent protection from rotenone-induced death by ATP depletion.

Bcl-2 Protects from STS-Induced Cytochrome c Release but Not from the Initial Drop of $\Delta\Psi_m$ Caused by STS in GSNO-Treated Cells

In contrast to rotenone, ATP depletion by GSNO did not lead to a loss of $\Delta\Psi_m$ and did not induce cytochrome *c* release by itself even at late time points (Figs. 5A and 5B). In both *neo* and *bcl-2* cells that were depleted of ATP by GSNO, $\Delta\Psi_m$ was slightly lower than that of the untreated respective controls, but did not decline further for up to 16 h (not shown). Small amounts of cytochrome *c* in cytosolic fractions of unstimulated *neo* cells were observed and correlated with a small subpopulation ($\sim$10%) that died following prolonged ATP depletion.

STS treatment under these conditions led to a complete and permanent loss of TMRE staining and to extensive cytochrome *c* release within 2–3 h in *neo* cells
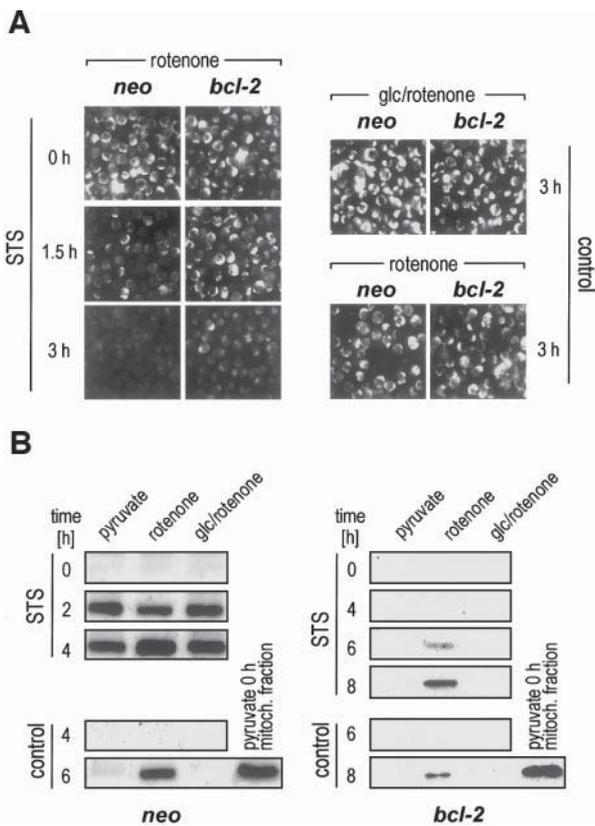
FIG. 4. Effects of Bcl-2 on STS-induced loss of the mitochondrial membrane potential $\Delta\Psi_m$ and cytochrome c release in rotenone-treated cells. A, $\Delta\Psi_m$ was visualized by using the potential sensitive dye TMRE. Jurkat *neo* and *bcl-2* cells were incubated in PNG medium with 0.2 μM rotenone in the presence or absence of glucose (glc) as indicated. (Left panels) Cells stimulated with 1 μM STS; (right panels) unstimulated control cells. At the given times, TMRE accumulation was recorded by video imaging. Each panel shows a field containing 45–60 cells and is representative for three independent experiments. B, Jurkat *neo* and *bcl-2* cells were incubated in PNG medium alone (pyruvate) or in the presence of 0.2 μM rotenone. Cells were exposed to 1 μM STS or solvent. Where indicated, medium was supplemented with 10 mM glucose (glc). At the given times, cytosolic fractions were isolated and analyzed for cytochrome c content by Western blot. For comparison, the mitochondrial fractions of either *neo* or *bcl-2* control cells (pyruvate 0 h), which represent total cellular cytochrome c, are shown. Western blot panels shown for each clone were from the same blot and from one of three representative experiments.

DISCUSSION

We addressed the question as to whether the survival advantage by Bcl-2 depends on normal ATP levels and whether Bcl-2 would also protect from death when apoptosis was converted to necrosis under conditions of energy loss.

Bcl-2 has varying effects on cell death caused by energy loss alone [26, 28, 35]. We focused here on conditions under which ATP depletion per se was not
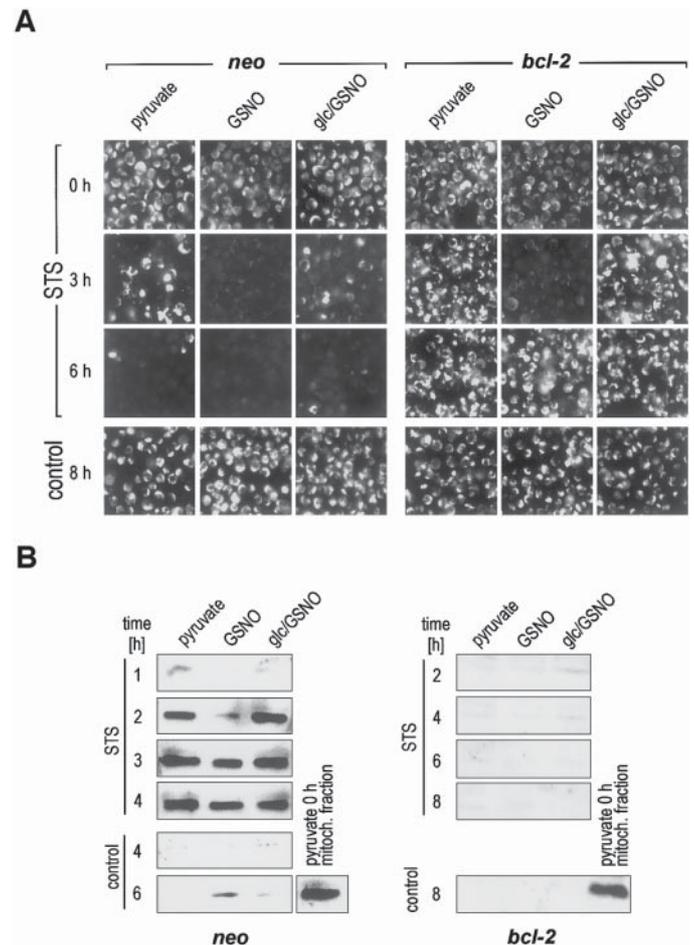
FIG. 5. Bcl-2 prevents cytochrome c release and restores STS-induced drop of the mitochondrial membrane potential $\Delta\Psi_m$ in cells depleted of ATP by GSNO. Jurkat *neo* and *bcl-2* cells were incubated in PNG medium (pyruvate) supplemented with glucose where indicated (glc). ATP was depleted by 0.4 mM GSNO. At 0 h, cells were exposed to 1 μM STS. A, $\Delta\Psi_m$ was visualized by using the potential sensitive dye TMRE. At the given times, TMRE accumulation was recorded by video imaging. Each panel shows a field containing 45–60 cells and is representative for three independent experiments. B, at the times indicated, cytosolic fractions were isolated and analyzed for cytochrome c content by Western blot. For comparison, the mitochondrial fractions of either *neo* or *bcl-2* control cells (pyruvate 0 h), which represent total cellular cytochrome c, are shown. Western blot panels shown for each clone were from the same blot and representative for three independent experiments.

(Figs. 5A and 5B). As seen in the rotenone model, Bcl-2 did not protect from the initial drop of $\Delta\Psi_m$ induced by STS. Nevertheless, cytochrome c release was completely inhibited, and *bcl-2* cells were able to recover $\Delta\Psi_m$ 4–6 h following STS treatment despite low intracellular ATP levels. The data suggest that maintenance of mitochondrial structural integrity by Bcl-2 may allow a recovery of $\Delta\Psi_m$ after an initial decrease due to a Bcl-2-independent mechanism in ATP-depleted cells.

lethal to the cells. In our experimental model, ATP depletion was well tolerated over a prolonged period of time (10 h when we used the strong mitochondrial poison rotenone and >18 h with the NO donor GSNO). This allowed us to mimic a situation in which energy-deprived cells are exposed to additional death stimuli. Such conditions are common *in vivo,* e.g., during ischemia in different tissues. In this model we could then investigate whether Bcl-2 counteracted the lethal effect of defined death triggers acting on ATP-depleted cells.

Both rotenone and GSNO switched STS-induced apoptosis to necrosis exclusively by inhibiting mitochondrial oxidative phosphorylation, whereas addition of glucose restored ATP levels and the execution of apoptosis [1, 3, 34]. Notably, the inhibition of apoptosis by NO in this system was not mediated by cGMP-dependent mechanisms, PARP activation, or direct inhibition of caspases [34]. Also, neither of the two ATP-depleting reagents affected the antiapoptotic function of Bcl-2 directly, because STS-induced caspase activation and apoptosis were prevented by Bcl-2 in the presence of either rotenone or NO *plus* glucose. Moreover, Bcl-2 did not influence ATP depletion by the different protocols.

In control vector-transfected cells, ATP depletion delayed STS-induced cytochrome *c* release by ∼1 h in accordance with data described earlier [3, 19]. However, after 3 h cytochrome *c* was released to the same extent in apoptosis and necrosis. Although high amounts of cytochrome *c* were found in the cytosol, ATP depletion persistently blocked the subsequent caspase activation [3, 19].

In general, intracellular death signalling often involves mitochondrial damage in both apoptosis and necrosis [36]. Once the mitochondria break, not only the proper mitochondrial function is lost, but also death-promoting proteins, e.g., cytochrome *c,* apoptosis-inducing factor, or caspases, are released [9, 37, 38]. Since the release of mitochondrial proteins is observed in both apoptosis and necrosis [3, 29, 39, 40], the shape of death may be decided by downstream events such as caspase activation, while the extent of cell death may be controlled by steps upstream or within mitochondria [36 and references therein]. Bcl-2 block of death pathways upstream of the point where apoptosis and necrosis signalling pathways diverge would therefore be most effective.

The protection by Bcl-2 from STS-induced death in cells with a high or low ATP level suggests that Bcl-2 acts upstream of the ATP-dependent step deciding the death mode. Moreover, the data show that Bcl-2 does not require high cellular ATP levels for this protection.

Although not all functions of Bcl-2 are known yet, four major mechanisms of cytoprotection have been described: (1) Prevention of caspase activation by inhi-

bition of the release of cytochrome *c* to the cytosol. This would prevent the proper assembly of the caspase activating apoptosome–complex [10, 11]. However, this mechanism can be ruled out here, because caspases are not involved in STS-induced necrosis in ATP-depleted cells. (2) Direct prevention of cytochrome *c* release and subsequent inhibition of an increased production of oxygen radicals by the respiratory chain [41]. (3) Stabilization of the mitochondria by preventing dissipation of the mitochondrial membrane potential $\Delta\Psi_m$, e.g., by facilitating proton exchange or by blocking permeability transition [42, 43]. (4) The inhibition of the release of additional mitochondrial proapoptotic factors, such as AIF, to prevent alternative death pathways [9].

The loss of $\Delta\Psi_m$ is frequently observed in apoptosis after the release of cytochrome *c* [10, 44–46], but it is unclear how these two events are connected. A decrease of $\Delta\Psi_m$ can occur in a reversible manner without any cytochrome *c* release and cell death [47–49]. ATP depletion alone does not necessarily lead to depolarization of the mitochondria [34]. On the contrary, inhibition of the mitochondrial ATP-synthetase, e.g., by oligomycin, results in mitochondrial hyperpolarization. When the electron transport is inhibited, as, e.g., here with rotenone or NO, $\Delta\Psi_m$ can still be maintained by reversed ATP-synthetase activity and hydrolysis of residual ATP [49]. Although Bcl-2 prevented both the decrease of $\Delta\Psi_m$ and the release of cytochrome *c* in STS-induced apoptosis, these two events are not necessarily related. For instance, in STS-induced necrosis, Bcl-2 did not protect from a decrease in $\Delta\Psi_m$ although cytochrome *c* release was inhibited. Therefore, one function of Bcl-2, namely the maintenance of high $\Delta\Psi_m$, might be ATP dependent, while the prevention of cytochrome *c* release by Bcl-2 seems to be independent of high cellular ATP levels. Since Bcl-2 protected from STS-induced death under ATP-depleting conditions without preventing a drop of $\Delta\Psi_m$, we conclude that the maintenance of $\Delta\Psi_m$ was not crucial for the protection by Bcl-2 from STS-induced necrosis.

When ATP was depleted with GSNO, Bcl-2 allowed cells to fully recover $\Delta\Psi_m$ within 6 h after the STS-induced drop. The recovery of $\Delta\Psi_m$ seemed to be important for the long-term retention of cytochrome *c* in the mitochondria and for long-term survival. Conversely, permanent loss of $\Delta\Psi_m$ by rotenone-mediated ATP depletion seemed to override Bcl-2 protection and led to cytochrome *c* release and cell death.

Apoptosis as well as necrosis occur in high frequency in pathological situations such as stroke, ischemia, organ transplantation, or inflammatory syndromes. Although these conditions are linked to impaired energy production, cell death under these conditions may not be a mere consequence of low intracellular ATP levels. Rather, the cells, already weakened by the lack of

energy, are confronted with the accumulation of potentially harmful catabolic endproducts and exposed to a cocktail of endogenously derived irritants and cytotoxins, such as reactive oxygen species or cytokines. Our data provide evidence that pharmacological upregulation of Bcl-2 can reduce not only apoptosis, but overall cell loss in such complex situations that would otherwise result in necrosis. Strategies to upregulate Bcl-2 function may therefore be more effective than those targeting only the downstream executors of apoptosis (i.e., caspases) under acute forms of injury when both apoptosis and necrosis are involved.

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