Effect of Sphingosine on Ca^{2+} Entry and Mitochondrial Potential of Jurkat T Cells – Interaction with Bcl_2

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Key Words

Apoptosis • Mitochondria • Capacitative Ca^{2+} channel I_{CRAC} • Jurkat cells

Abstract

Triggers of Jurkat T cell apoptosis include sphingosine and ceramide. Sphingosine and ceramide further inhibit capacitative Ca^{2+} entry (I_{CRAC}), an effect leading to inactivation but not death of Jurkat T cells. Mitochondria are key organelles in the machinery leading to apoptosis and on the other hand have been shown to participate in the regulation of Ca^{2+} entry. The present experiments were performed to explore whether treatment of Jurkat T cells with sphingosine leads to apoptosis and reduced Ca^{2+} entry and whether those effects are sensitive to expression of the antiapoptotic protein Bcl_2 , localized in the outer mitochondrial membrane. Exposure of Jurkat T cells to 10 μM sphingosine was according to DiOC_6 fluorescence followed by mitochondrial depolarization and according to Fura-red/Fluo-3 fluorescence followed by decreased capacitative Ca^{2+} entry. Mitochondrial depolarization was significantly delayed in cells overexpressing wild type Bcl_2 or Bcl_2 targeted to the mitochondrial membrane, whereas no

significant influence on mitochondrial depolarization was observed in cells expressing Bcl_2 lacking the membrane targeting motif or Bcl_2 targeted to the endoplasmatic reticulum. In contrast to mitochondrial potential, the blunting of capacitative Ca^{2+} entry following sphingosine treatment was not sensitive to mitochondrial Bcl_2 expression. In conclusion sphingosine exposure leads to both, mitochondrial depolarization and inhibition of capacitative Ca^{2+} entry. Mitochondrial Bcl_2 reverses the effect on mitochondria but not on Ca^{2+} entry and thus leads to dissociation of those two sequelae of sphingosine treatment.

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Introduction

Apoptosis is a physiological mechanism leading to DNA fragmentation, cell shrinkage, mitochondrial depolarization, caspase activation and phosphatidylserine exposure at the cell surface [1-23]. Triggers of Jurkat cell apoptosis include sphingosine [24, 25] and ceramide [26-30]. Sphingosine [31] and ceramide [32, 33] further inhibit capacitative Ca^{2+} entry (I_{CRAC}), an effect leading to inactivation but not death of Jurkat T cells [33].

As mitochondria are thought to participate in the

regulation of Ca^{2+} entry [34–36], the inhibition of Ca^{2+} entry following ceramide or sphingosine treatment could be due to mitochondrial depolarization. Alternatively, mitochondrial depolarisation and I_{CRAC} inhibition could be two separate, sphingosine sensitive events.

Mitochondrial depolarization could be prevented by expression of Bcl_2 , a protein known to protect cells from apoptotic cell death [37–45]. Bcl_2 is attached to the outer mitochondrial membrane, to endoplasmic reticulum membranes and to the nuclear envelope [6]. Mechanisms involved in the antiapoptotic action of Bcl_2 include inhibition of cytochrome c release from mitochondria which otherwise binds to Apaf-1 and activates caspase 9 thus triggering a caspase cascade [6, 42, 46, 47].

The present study was performed to explore whether Bcl_2 expression interferes with mitochondrial depolarization and inhibition of capacitative Ca^{2+} entry (I_{CRAC}) following treatment of Jurkat T cells with sphingosine.

Materials and Methods

Solutions and chemicals

Experiments were performed at 37°C in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO_4 , 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl_2 ; pH 7.4. Where indicated, sphingosine (10 μM , Biomol, Hamburg, Germany) and ionomycin (10 μM , Calbiochem, Bad Soden, Germany) and thapsigargin (1 μM , Molecular Probes Gottingen, Germany) were applied at the concentrations indicated. Dimethylsulfoxide was used as solvent at concentrations <0.1%.

Cell Culture

Human Jurkat T cells were transfected with Bcl_2 variants and subcellular locations verified as described previously [48]. In short, expression vectors encoding wild-type Bcl_2 (Bcl_2 /WT), cytoplasmic Bcl_2 lacking the transmembrane domain (Bcl_2 /DTM) and Bcl_2 mutants restricted to the outer mitochondrial membrane (Bcl_2 /MT) or endoplasmic reticulum (Bcl_2 /ER) were kindly provided by B. Leber (Ontario, Canada). Mitochondrial and ER-specific targeting of Bcl_2 was achieved by exchanging the C-terminal insertion sequence of Bcl_2 with equivalent sequences of the listerial protein ActA and rat cytochrome b5, respectively. Jurkat cells stably expressing the different versions of Bcl_2 and the respective vector control were prepared by electroporation.

Transfected and nontransfected cells were maintained in an atmosphere containing 5 % CO_2 at 37 °C in RPMI-1640 Medium supplemented with 10 % (v/v) heat-inactivated foetal calf serum, 100 μM non-essential amino acids, 2 mM L-glutamine, 100 U/ml Penicillin, 100 $\mu\text{g}/\text{ml}$ Streptomycin, and 50 μM β -Mercaptoethanol (all substances from Life Technologies,

Fig. 1. Decrease of mitochondrial potential following sphingosine treatment. Histograms of DiOC_6 fluorescence reflecting the mitochondrial potential in control cells (shaded area) and in cells pretreated with 10 M sphingosine for 2 hours (A) or 4 hours (B) (black line). At the left histogram of cells exposed to the mitochondrial poison CCCP (50 μM) as positive control.

EGgenstein, Germany). After 48 h, cells were exposed to sphingosine at the indicated concentrations for 12 h. Thereafter cells were harvested and analyzed for Fura-2 imaging.

Measurement of intracellular Ca^{2+} activity

Fura-Red and Fluo-3 fluorescence were utilized for cytosolic Ca^{2+} determinations [49, 50]. Intracellular Ca^{2+} measurements were performed by FACS analysis as described [33] at an excitation wavelength of 488 nm and emission wavelengths of 650 nm and 530 nm. Prior to the experiments Jurkat T cells were loaded with Fura-Red-AM (4.5 μM , Molecular Probes, Gottingen, Germany) for 30 minutes and Fluo3-AM (0.5 μM , Molecular Probes, Gottingen, Germany) for 10 minutes at 37°C. Calibration was achieved by exposing the cells in the presence of Ca^{2+} ionophore ionomycin (10 μM) to 2 mM extracellular Ca^{2+} concentration or to 2 mM EGTA in the absence of Ca^{2+} . The intracellular Ca^{2+} stores were depleted by inhibition of the vesicular Ca^{2+} pump by thapsigargin (1 μM , Molecular Probes Gottingen, Germany).

Determination of mitochondrial potential

The mitochondrial potential was determined using the fluorescent dye DiOC_6 (Molecular Probes, Eugene, USA) in FACS analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Prior to the experiments cells were loaded with DiOC_6 (0.2 nM- Molecular Probes, Gottingen, Germany) for 30 minutes at 37°C.

Statistics

Data are expressed as arithmetic means $\pm$ SEM and statistical analysis was made by paired or unpaired t-test, where appropriate.

Fig. 2. Decrease of capacitative Ca^{2+} entry following sphingosine treatment. Histograms of cytosolic Ca^{2+} concentrations (fluorescence ratio in arbitrary units) of cells treated with $1\ \mu\text{M}$ thapsigargin in the presence of extracellular Ca^{2+} without (open columns) or with (closed columns) pretreatment with $10\ \mu\text{M}$ sphingosine for 2 hours (A) or 4 hours (B).

Results

As illustrated in Fig. 1, exposure of Jurkat T cells to $10\ \mu\text{M}$ sphingosine was followed by mitochondrial depolarization. Within two hours treatment with sphingosine the mitochondria were depolarized in approximately 1/3 of the cells, after a 4 hours treatment with sphingosine mitochondria were depolarized in some 2/3 of the cells.

The treatment with sphingosine further decreased capacitative Ca^{2+} entry. As illustrated in Fig. 2 cytosolic Ca^{2+} concentrations following treatment with $1\ \mu\text{M}$ thapsigargin in the presence of extracellular Ca^{2+} were significantly lower in cells pretreated with $10\ \mu\text{M}$ sphingosine for 2 or 4 hours.

The expression of Bcl_2 in the mitochondrial membrane delayed mitochondrial depolarization following sphingosine treatment. Histograms of DiOC_6 fluorescence (Fig. 3) again demonstrate the marked decrease of the mitochondrial potential in control cells following treatment with $10\ \mu\text{M}$ sphingosine for 2 or 4 hours. The mitochondrial depolarization was significantly delayed in cells overexpressing Bcl_2 in the mitochondrial membrane (ACTA and WT), whereas no significant influence on mitochondrial depolarization was observed in cells expressing Bcl_2 outside of mitochondria (CB5 and ΔTM).

In contrast to mitochondrial potential, the blunting of capacitative Ca^{2+} entry following sphingosine treatment was not sensitive to mitochondrial Bcl_2 expression (Fig. 4). Histograms of fluorescence ratios demonstrate a decrease of cytosolic Ca^{2+} concentrations following pretreatment with $10\ \mu\text{M}$ sphingosine for 2 hours, irrespective of Bcl_2 expression. The decrease was similar in vector control cells (VC), in cells overexpressing Bcl_2 in the mitochondrial membrane (Acta and WT) and in cells expressing Bcl_2 outside of mitochondria (CB5 and ΔTM).

Fig. 3. Bcl_2 in the mitochondrial membrane delays mitochondrial depolarization following sphingosine treatment. Histograms of DiOC_6 fluorescence reflecting the mitochondrial potential in control cells (shaded area) and in cells pretreated with $10\ \mu\text{M}$ sphingosine (black lines) for 2 hours (A) or 4 hours (B). Experiments were performed in vector control cells (VC), in cells overexpressing Bcl_2 in the mitochondrial membrane (Acta and WT) and in cells expressing Bcl_2 outside of mitochondria (CB5 and ΔTM).

Fig. 4. Mitochondrial Bcl₂ expression does not blunt the decrease of capacitative Ca²⁺ entry following sphingosine treatment. Histograms of cytosolic Ca²⁺ concentrations (fluorescence ratio in arbitrary units) of cells treated with 1 μ M thapsigargin in the presence of extracellular Ca²⁺ without (open columns) or with (closed columns) pretreatment with 10 μ M sphingosine for 2 hours. Experiments were performed in vector control cells (VC), in cells overexpressing Bcl₂ in the mitochondrial membrane (Acta and WT) and in cells expressing Bcl2 outside of mitochondria (CB5 and Δ TM).

Discussion

The present study confirms the previous observations demonstrating the ability of sphingosine to trigger apoptosis of Jurkat cells [27, 28] and to inhibit capacitative Ca²⁺ entry (I_{CRAC}) into Jurkat T cells [31, 33]. The stimulation of apoptosis is apparent from mitochondrial depolarization, a key event in apoptotic cell death [6, 46, 51-56], the inhibition of I_{CRAC} is evidenced by delayed entry of Ca²⁺ into thapsigargin treated cells, which parallels the electrophysiology [33, 57-60]. Sphingosine could be converted to ceramide, a reversible reaction catalyzed by ceramidase [61, 62]. Thus, the effect of sphingosine could either be direct or through formation of ceramide.

Mitochondrial depolarization was significantly delayed in cells overexpressing wild type Bcl₂ or Bcl₂ targeted to the mitochondrial membrane (ACTA), confirming the protective role of mitochondrial Bcl₂ [37]. On the other hand, mitochondrial depolarization upon treatment with sphingosine was not appreciably modified in cells expressing Bcl₂ outside of mitochondria. Thus, mitochondrial localization is required for the protective effect of Bcl₂, an observation in line with the known mechanism of action [37-45].

The present experiments further demonstrate that treatment of Jurkat T lymphocytes with sphingosine decreases the slope of capacitative Ca²⁺ entry (I_{CRAC})

which is indicative for an inactivation of store operated Ca²⁺ channels [54, 63-65].

A wide variety of hormones and mediators activate I_{CRAC} including mitogens and inflammatory cytokines [33, 65, 66]. Altered regulation of cytosolic Ca²⁺ is expected to affect diverse cellular functions, such as control of exocytosis, contraction, enzyme activity and gene expression [65, 67-69]. Moreover, activation of I_{CRAC} has been shown to stimulate cell proliferation [70], inhibition of I_{CRAC} parallels apoptotic death and inactivation of Jurkat lymphocytes [33]. All those cellular functions are expected to be sensitive to sphingosine treatment.

In contrast to mitochondrial potential, the blunting of capacitative Ca²⁺ entry following sphingosine treatment was not sensitive to mitochondrial Bcl₂ expression. Thus, mitochondrial depolarization is obviously not required for the inhibition of I_{CRAC} . Apparently, sphingosine influences mitochondrial potential and I_{CRAC} activity by two distinct mechanisms. Even though the observation does not rule out that the two effects share upstream signaling pathways, they clearly show that mitochondrial depolarization does not account for the inhibition of I_{CRAC} . Notably, Bcl₂ targeting to endosomal vesicles similarly remained without influence on I_{CRAC} indicating that Bcl₂

is similarly unable to modify Ca^{2+} release from those stores.

In conclusion, in Jurkat T lymphocytes sphingosine triggers two separate cellular functions, i.e. mitochondrial depolarization and inhibition of capacitative Ca^{2+} entry I_{CRAC} . The former effect leads to apoptosis, the latter blocks activation. Mitochondrial but not cytosolic or endosomal Bcl_2 interferes with mitochondrial depolarization whereas neither mitochondrial, cytosolic nor endosomal Bcl_2 significantly modifies I_{CRAC} . Accordingly,

mitochondrial depolarization and inhibition of I_{CRAC} are two independent effects of sphingosine treatment.

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