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Bcl-x_L knockout attenuates mitochondrial respiration and causes oxidative stress that is compensated by pentose phosphate pathway activity

Annika Pfeiffer[a], Julia Schneider[a], Diones Bueno[a], Amalia Dolga[b], Timo-Daniel Voss[c], Jan Lewerenz[c], Verena Wüllner[a], Axel Methner[a,*]

[a] Department of Neurology, University Medical Center and Focus Program Translational Neuroscience (FTN) of the Johannes Gutenberg-University Mainz, Langenbeckstr. 1, 55131 Mainz, Germany
[b] Department of Molecular Pharmacology, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands
[c] Universitäts, und Rehabilitationskliniken Ulm, Oberer Eselsberg 45, 89081 Ulm, Germany

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ABSTRACT

Bcl-x_L is an anti-apoptotic protein that localizes to the outer mitochondrial membrane and influences mitochondrial bioenergetics by controlling Ca^{2+} influx into mitochondria. Here, we analyzed the effect of mitochondrial Bcl-x_L on mitochondrial shape and function in knockout (KO), wild type and rescued mouse embryonic fibroblast cell lines. Mitochondria of KO cells were more fragmented, exhibited a reduced ATP concentration, and reduced oxidative phosphorylation (OXPHOS) suggesting an increased importance of ATP generation by other means. Under steady-state conditions, acidification of the growth medium as a readout for glycolysis was similar, but upon inhibition of ATP synthase with oligomycin, KO cells displayed an instant increase in glycolysis. In addition, forced energy production through OXPHOS by replacing glucose with galactose in the growth medium rendered KO cells more susceptible to mitochondrial toxins. KO cells had increased cellular reactive oxygen species and were more susceptible to oxidative stress, but had higher glutathione levels, which were however more rapidly consumed under conditions of oxidative stress. This coincided with an increased activity and protein abundance of the pentose phosphate pathway protein glucose-6-phosphate dehydrogenase, which generates NADPH necessary to regenerate reduced glutathione. KO cells were also less susceptible to pharmacological inhibition of the pentose phosphate pathway. We conclude that mitochondrial Bcl-x_L is involved in maintaining mitochondrial respiratory capacity. Its deficiency causes oxidative stress, which is associated with an increased glycolytic capacity and balanced by an increased activity of the pentose phosphate pathway.

1. Introduction

The yin and yang of life and death is regulated to a significant degree by members of the Bcl-2 (B-cell lymphoma protein 2) family, which regulate the execution of apoptosis, the cell death program essential for normal development and homeostasis of a multicellular organism (reviewed by [1]). The two most prominent anti-apoptotic family members, Bcl-2 and Bcl-x_L (Bcl-2-related protein, long isoform), share a common structure with four conserved Bcl-2 homology domains and a C-terminal membrane anchor, but differ in their subcellular localization. Bcl-2 distributes on several intracellular membranes, whereas Bcl-x_L is mainly targeted to the outer mitochondrial membrane (OMM) [2]. Both proteins interact with the inositol 1,4,5-triphosphate (InsP3) receptor (InsP3R) Ca^{2+} release channel at the endoplasmic reticulum (ER), although with different domains and with opposite effects. Whereas Bcl-2 reduces the InsP3R-mediated intracellular Ca^{2+} release [3–5], Bcl-x_L sensitizes InsP3Rs to low agonist concentrations, which stimulates Ca^{2+} oscillations and Ca^{2+} flux into the cytosol, thereby enhancing mitochondrial bioenergetics [6,7]. In mouse embryonic fibroblasts (MEFs) derived from *bcl-x* knockout (KO) animals,

Abbreviations: 2-DG, 2-Deoxy-D-glucose; ADP, adenosine diphosphate; Ama, antimycin A; ATP, adenosine triphosphate; c, cytochrome c; Bcl-x_L, B-cell lymphoma protein 2-related protein, long isoform; dig, digitonin; E, electron transfer system capacity state; ECAR, extracellular acidification rate; ER, endoplasmic reticulum; ETS, electron transfer system; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; FCS, fetal calf serum; G, glutamate; GSH, reduced glutathione; GSSG, glutathione disulfide/oxidized glutathione ICC, immunocytochemistry; KO, knockout; L, leak state;; M, malate; NAD, nicotinamide adenine dinucleotide; OMM, outer mitochondrial membrane; Omy, oligomycin; OXPHOS, oxidative phosphorylation; P, OXPHOS capacity state; PPP, pentose phosphate pathway; R, routine state; Rot, rotenone; ROX, residual oxygen consumption; RT, room temperature; S, succinate; SUIT, substrate-uncoupler-inhibitor titration; PCP, phosphorylation-control protocol

* Correspondence to: Johannes Gutenberg University Medical Center Mainz, Department of Neurology, Langenbeckstr. 1, D-55131 Mainz, Germany.
E-mail address: axel.methner@gmail.com (A. Methner).

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which also lack the pro-apoptotic alternative splice variant Bcl-x_s, only re-expression of mitochondrially targeted, but not ER-localized Bcl-x_L, restored protection against anti-apoptotic stimuli to levels observed in wildtype (WT) cells [8]. ER-targeted Bcl-x_L, in contrast, restored the ER Ca^{2+} homeostasis suggesting two independent functions of Bcl-x_L, depending on localization to the ER or the mitochondria [8].

Bcl-x_L also affects the constantly ongoing mitochondrial fusion and fission that keeps mitochondria healthy through the exchange of mitochondrial DNA, proteins and lipids between defective and functional mitochondria. These highly dynamic processes are important for mitochondrial bioenergetics and cellular survival (reviewed in [9]). In rat cortical neurons over-expressing Bcl-x_L, mitochondria are elongated, and in knockout neurons more fragmented [10]. Bcl-x_L influences mitochondrial dynamics by interacting with the mitochondrial fusion proteins mitofusin 1 (Mfn1) and 2 [11], as well as with the mitochondrial fission factor Drp-1 [12], by increasing their respective functions. It was postulated that Bcl-x_L promotes both mitochondrial fusion and fission dependent on the relative protein expression level of Bcl-x_L [13,14].

Bcl-x_L also affects mitochondrial function. Over-expression in hippocampal neurons caused an enhanced and more efficient energy metabolism, based on an increase in ATP production and decreased mitochondrial oxygen uptake, reflecting a more efficient coupling between ATP production and oxygen uptake [15]. Here, the proposed mechanism postulated an additional localization of Bcl-x_L in the inner mitochondrial membrane and a direct interaction with the β-subunit of the F_1F_o ATP synthase [15]. In addition, neurons deficient for *bcl-x* displayed large fluctuations in inner mitochondrial membrane potential, indicating increased total ion flux in and out of mitochondria and suggesting a role for Bcl-x_L in the reduction of futile ion flux across the inner mitochondrial membrane [16]. An alternative mechanism of how Bcl-x_L affects the mitochondrial function involves the interaction of Bcl-x_L with voltage-dependent anion channels (VDAC) at the OMM [17,18]. VDAC channels are responsible for the regulated crossing of metabolites and Ca^{2+} through the membrane and are of utmost importance for the correct functioning of mitochondria [19]. Recently, Huang et al. found a reduced mitochondrial Ca^{2+} uptake in the same Bcl-x-KO cells described above in response to agonist-induced ER Ca^{2+} release, which was restored by re-expression of mitochondrially targeted Bcl-x_L [20]. Interestingly, peptides based on the VDAC sequence that disrupted Bcl-x_L binding also reduced mitochondrial Ca^{2+} uptake in wild type (WT), but were without effect in Bcl-x-KO cells. The authors concluded that an interaction between Bcl-x_L and VDAC promotes matrix Ca^{2+} accumulation by increasing Ca^{2+} transfer across the OMM. As elevated mitochondrial matrix [Ca^{2+}] stimulates Krebs' cycle dehydrogenases and elevates mitochondrial [NADH] that is fed into the oxidative phosphorylation pathway [6], this should increase mitochondrial respiration. Therefore, we expected that Bcl-x_L deficiency influences mitochondrial respiratory activity and changes mitochondrial morphology.

Here, we performed an in-depth analysis of the effect of mitochondrial Bcl-x_L on mitochondrial shape and function in well-characterized Bcl-x-KO, WT and rescue MEF cell lines. We found that mitochondrial Bcl-x_L controls mitochondrial respiratory capacity and ATP production. Its deficiency leads to more fragmented mitochondria, an increase in glycolytic capacity and an increase in non-mitochondrial oxidative stress, which is compensated by increased activity of the pentose phosphate pathway.

2. Material and methods

2.1. Cell culture

The MEF cell lines WT, Bcl-x-KO and Bcl-x_L-ActA (kind gift of Carl White, Chicago, USA) [8] were cultured in DMEM high glucose (#E15-843, PAA, Pasching, Austria; #D6429, Sigma-Aldrich, Steinheim, Germany; or #41966029, Gibco, Paisley, United Kingdom) supplemented with 10% (v/v) fetal calf serum (FCS; Thermo Scientific, Rockford, IL, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) in a humidified incubator at 5% CO_2 and 95% air at 37 °C. Bcl-x_L-ActA MEFs stably over-express a Bcl-x_L where the C-terminal transmembrane sequence is replaced by the membrane-targeting sequence of the listerial protein ActA to target Bcl-x_L specifically to mitochondria [8]. These cells were grown in medium supplemented with 1.5 µg/ml blasticidin (#ant-bl-1, InvivoGen, Toulouse, France).

2.2. Immunoblotting

Denatured total cellular protein samples in RIPA buffer (#89900, Thermo Scientific) supplemented with 1x LDS (#NP007, Invitrogen) or Laemmli sample buffer (#1610747, BioRad) (95 °C, 5 min) were separated on SDS polyacrylamide gels (8–16% Precise Protein Gels (Thermo Scientific) or 4–15% Mini-PROTEAN® TGX Stain-Free™ gels (Bio-Rad Laboratories GmbH, München, Germany) and transferred onto a membrane (nitrocellulose or PVDF) using the iBlot Dry Blotting System (Invitrogen, Darmstadt, Germany) or Trans-Blot® Turbo™ Transfer System (Bio-Rad). Membranes were blocked with 3% (w/v) milk powder in PBS-T (1x PBS, 0.05% (v/v) Tween 20, blocking buffer) for 1 h at room temperature (RT). The membrane was incubated at 4 °C overnight with the primary antibodies anti-Bcl-$x_{S/L}$ (S-18) (1:100–1:200; #sc-634, Santa Cruz Biotechnology, Heidelberg, Germany), anti-G6PD (D5D2) (1:1000; #12263, Cell Signaling, Frankfurt, Germany), anti-actin (clone C4) (1:4000; #MAB1501, Merck Millipore, Darmstadt, Germany) in blocking buffer. For visualization, membranes were incubated with infrared fluorescence IRDye 680 and 800-conjugated anti-mouse/anti-rabbit IgG secondary antibodies (1:30,000; Licor, Königstein, Germany) in blocking buffer for 1 h at RT and detected with the Odyssey Infrared (Sa) Imaging System (Licor). The software ImageJ (http://imagej.nih.gov/ij/) was used to analyze the expression of proteins in relation to the control.

2.3. Immunocytochemistry

Cells transfected with pDsRed2-mito were seeded onto cover slips and fixed the next day with 4% Roti®-Histofix (15 min at RT) (#P087.4, Carl Roth, Karlsruhe, Germany), permeabilized and blocked with 1x ROTI-Immunoblock in PBS (#T144.1, Carl Roth) supplemented with 0.5% (w/v) n-octyl-β-D-glucopyranoside (Carl Roth; ICC blocking buffer) (60 min at RT). The primary antibody anti-Bcl-x_L (54H6) (#2764, Cell Signaling) was diluted 1:200 in the ICC blocking buffer and cover slips were incubated at 4 °C overnight. The fluorescein isothiocyanate (FITC)-conjugated secondary antibody was diluted in the same ICC blocking buffer and cells were incubated for 1 h at RT before cell nuclei were stained with 300 nM DAPI (5 min, RT). Cover slips were mounted with Dako Fluorescent Mounting Medium (#S3023, Dako, Glostrup, Denmark) onto microscope slides and fluorescence pictures were taken with the same settings from one optical section using a 63× oil immersion objective with the confocal microscope TCS SP5 from Leica (Leica Microsystems, Wetzlar, Germany).

2.4. Analysis of the mitochondrial morphology

Cells were transfected with mitochondrially targeted DsRed2, a red fluorescent protein, using the Attractene Transfection Reagent (#301005, Qiagen, Hilden, Germany) according to the manufacturer's instruction. One day after transfection, cells were seeded onto cover slips. The next day, cells were fixed with 4% Roti®-Histofix for 15 min at RT, permeabilized with 0.2% (v/v) Triton X-100 in PBS for 10 min and then cell nuclei were stained with 300 nM DAPI (5 min, RT). Cover slips were mounted onto microscope slides using Dako Fluorescence Mounting Medium. Cells were categorized by a blinded observer and representative pictures were taken with an Olympus BX51 fluorescence