Free Radical Biology & Medicine 52 (2012) 410–419

Contents lists available at SciVerse ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Bcl-2 is a novel interacting partner for the 2-oxoglutarate carrier and a key regulator of mitochondrial glutathione

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ARTICLE INFO

Article history:
Received 1 July 2011
Revised 28 October 2011
Accepted 31 October 2011
Available online 9 November 2011

Keywords:
Reduced glutathione
Mitochondrial oxidative stress
Bcl-2
2-Oxoglutarate carrier
Free radicals

ABSTRACT

Despite making up only a minor fraction of the total cellular glutathione, recent studies indicate that the mitochondrial glutathione pool is essential for cell survival. Selective depletion of mitochondrial glutathione is sufficient to sensitize cells to mitochondrial oxidative stress (MOS) and intrinsic apoptosis. Glutathione is synthesized exclusively in the cytoplasm and must be actively transported into mitochondria. Therefore, regulation of mitochondrial glutathione transport is a key factor in maintaining the antioxidant status of mitochondria. Bcl-2 resides in the outer mitochondrial membrane where it acts as a central regulator of the intrinsic apoptotic cascade. In addition, Bcl-2 displays an antioxidant-like function that has been linked experimentally to the regulation of cellular glutathione content. We have previously demonstrated a novel interaction between recombinant Bcl-2 and reduced glutathione (GSH), which was antagonized by either Bcl-2 homology-3 domain (BH3) mimetics or a BH3-only protein, recombinant Bim. These previous findings prompted us to investigate if this novel Bcl-2/GSH interaction might play a role in regulating mitochondrial glutathione transport. Incubation of primary cultures of cerebellar granule neurons (CGNs) with the BH3 mimetic HA14-1 induced MOS and caused specific depletion of the mitochondrial glutathione pool. Bcl-2 was coimmunoprecipitated with GSH after chemical cross-linking in CGNs and this Bcl-2/GSH interaction was antagonized by preincubation with HA14-1. Moreover, both HA14-1 and recombinant Bim inhibited GSH transport into isolated rat brain mitochondria. To further investigate a possible link between Bcl-2 function and mitochondrial glutathione transport, we next examined if Bcl-2 associated with the 2-oxoglutarate carrier (OGC), an inner mitochondrial membrane protein known to transport glutathione in liver and kidney. After cotransfection of CHO cells, Bcl-2 was coimmunoprecipitated with OGC and this novel interaction was significantly enhanced by glutathione monoethyl ester. Similarly, recombinant Bcl-2 interacted with recombinant OGC in the presence of GSH. Bcl-2 and OGC cotransfection in CHO cells significantly increased the mitochondrial glutathione pool. Finally, the ability of Bcl-2 to protect CHO cells from apoptosis induced by hydrogen peroxide was significantly attenuated by the OGC inhibitor phenylsuccinate. These data suggest that GSH binding by Bcl-2 enhances its affinity for the OGC. Bcl-2 and OGC appear to act in a coordinated manner to increase the mitochondrial glutathione pool and enhance resistance of cells to oxidative stress. We conclude that regulation of mitochondrial glutathione transport is a principal mechanism by which Bcl-2 suppresses MOS.

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Mitochondrial oxidative stress (MOS)[1] plays a key role in the pathology underlying several neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), Parkinsons disease, and Alzheimers disease [1]. Accordingly, elucidating the pathways that regulate the mitochondrial oxidant/antioxidant balance is essential to develop novel therapeutics for neurodegeneration. Glutathione is an endogenous tripeptide antioxidant and a key player in averting MOS and evading apoptosis [reviewed in 2]. It has been previously shown that selective depletion of mitochondrial glutathione sensitizes cells to oxidative or nitrosative stress [3,4]. Moreover, glutathione depletion can induce apoptosis directly through opening of the

Abbreviations: BH3, Bcl-2 homology-3 domain; CGN, cerebellar granule neuron; CHO, Chinese hamster ovary cells; CNS, central nervous system; DIC, dicarboxylate carrier; DMSO, dimethyl sulfoxide; DSS, disuccinimidyl suberate; DTT, dithiothreitol; GSH, reduced glutathione; GSH-MEE, glutathione monoethyl ester; GST, glutathione *S*-transferase; HA14-1, 2-amino-6-bromo-α-cyano-3-(ethoxycarbonyl)-4*H*-1-benzopyran-4-acetic acid ethyl ester; IMM, inner mitochondrial membrane; MOS, mitochondrial oxidative stress; OGC, 2-oxoglutarate carrier; PBS, phosphate-buffered saline; PhS, phenylsuccinate; SOD1, Cu/Zn superoxide dismutase.

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0891-5849/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.freeradbiomed.2011.10.495

mitochondrial permeability transition pore [5]. In addition, γ-glutamylcysteine synthetase knockout mice, in which glutathione synthesis is inhibited and glutathione is depleted, display significant apoptotic cell death in multiple tissues [6]. These findings demonstrate that maintenance of cellular glutathione and, in particular, the mitochondrial glutathione pool is crucial for cell survival [7,8].

Glutathione synthesis occurs exclusively in the cytosol because the enzymes required for its synthesis are absent within mitochondria [9]. Furthermore, glutathione has an overall negative charge at physiological pH and mitochondria exhibit a large negative membrane potential; consequently, glutathione transport into mitochondria cannot be explained by simple diffusion [9,10]. Previously, two inner mitochondrial membrane (IMM) anion carriers were identified in kidney and liver as glutathione transporters, the 2-oxoglutarate carrier (OGC; *Slc25a11*) and the dicarboxylate carrier (DIC; *Slc25a10*) [11-14]. Overexpression of either OGC or DIC in a rat renal proximal tubular cell line (NRK-52E cells) significantly enhanced mitochondrial glutathione transport and protected these cells from chemically induced apoptosis, such as that induced by *tert*-butylhydroperoxide [15,16].

In the context of the CNS, few studies have examined the mechanisms responsible for mitochondrial glutathione transport. In one study, glutathione transport into isolated rat brain mitochondria seemed to be influenced most by inhibitors of the tricarboxylate carrier rather than OGC or DIC [17]. However, another study showed that an inhibitor of DIC, butylmalonate, significantly decreased the glutathione content of isolated mouse brain mitochondria, suggesting that DIC may be the major glutathione transporter in mouse cerebral cortical mitochondria [18]. The authors of this study also showed that both OGC and DIC are expressed in cortical neurons and astrocytes. These studies suggest that multiple IMM anion transporters might be involved in mitochondrial glutathione transport in the CNS. Yet, essentially nothing is known about how the function of these IMM glutathione transporters is regulated.

Previous studies have shown that the antiapoptotic protein Bcl-2 displays an antioxidant-like effect in response to either exogenous oxidative stress or glutathione depletion [19,20]. Overexpression of Bcl-2 leads to an increase in the cellular content of glutathione [21,22]. In contrast, Bcl-2 knockout mice show reduced glutathione levels and glutathione peroxidase activity in brain tissue and demonstrate enhanced susceptibility to MOS-induced neuronal cell death [23]. Thus, the antioxidant-like function of Bcl-2 depends, in large part, on its potential to regulate the cellular glutathione status. In this context, we have previously shown that recombinant Bcl-2 is capable of directly binding to GSH in vitro, an interaction that is antagonized by the Bcl-2 homology-3 domain (BH3) mimetics HA14-1 and compound 6, as well as the BH3-only protein, Bim [24]. Interestingly, several BH3-only proteins are known to induce a pro-oxidant state at mitochondria, suggesting that disruption of this Bcl-2/GSH interaction might be an underlying factor in this effect [25,26].

Collectively, these findings prompted us to hypothesize that Bcl-2 might be a key regulator of the mitochondrial glutathione pool. Here, we show that Bcl-2 interacts with GSH in intact primary cerebellar granule neurons (CGNs). As we have shown previously using recombinant proteins, this Bcl-2/GSH interaction is disrupted by the BH3 mimetic HA14-1. Consistent with a central role for Bcl-2 in maintenance of the mitochondrial glutathione pool, both HA14-1 and Bim inhibited mitochondrial GSH transport. Most significantly, in cotransfected CHO cells, Bcl-2 coimmunoprecipitates with OGC and this novel interaction is markedly enhanced by glutathione monoethyl ester (GSH-MEE). Moreover, Bcl-2 and OGC coexpression significantly increases the mitochondrial glutathione pool. Finally, we show that the ability of Bcl-2 to protect cells from apoptosis induced by hydrogen peroxide depends on OGC activity. We conclude that Bcl-2 is a novel interacting partner for OGC and a central regulator of the mitochondrial glutathione pool. This newly discovered property of Bcl-2 suggests a molecular mechanism by which Bcl-2 protects cells from oxidative injury.

Materials and methods

Materials

Ethacrynic acid, Triton X-100, DTT, anti-tubulin antibody, DAPI, and phenylsuccinic acid were received from Sigma–Aldrich (St. Louis, MO, USA). HA14-1 was purchased from Alexis Biochemicals (Enzo Life Sciences, Plymouth Meeting, PA, USA). Glutathione assay kit was obtained from Oxford Biomedical (Rochester Hills, MI, USA). Mitochondrial–Cytosolic fractionation kit was purchased from Biovision (Mountain View, CA, USA). MDA assay kit was obtained from OxisResearch (Percipio Bioscience, Foster City, CA, USA). Anti-Cox-IV was purchased from Cell Signaling (Beverly, MA, USA). Glutathione monoethyl ester and rotenone were received from Calbiochem (San Diego, CA, USA). Anti-V5 antibody was purchased from Abcam (Cambridge, MA, USA). Recombinant BimL was obtained from R&D Systems (Minneapolis, MN, USA). The anti-GSH antibody and protein A/G beads were received from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Disuccinimidyl suberate (DSS) was purchased from Pierce Chemical (Rockford, IL, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Anti-Bcl-2 antibody was from BD Pharmingen (Franklin Lakes, NJ, USA). Mouse TrueBlot Ultra anti-mouse Ig–horseradish peroxidase (HRP) secondary antibody was obtained from eBioscience (San Diego, CA, USA). GST-tagged Slc25a11 (GST–OGC) recombinant protein and Slc25a11 (OGC) antibody were purchased from Novus Biologicals (Littleton, CO, USA). Recombinant Bcl-2 protein was obtained from Calbiochem (Darmstadt, Germany). Opti-MEM medium was purchased from Gibco (Carlsbad, CA, USA). Maxiprep Kit was obtained from Qiagen (Valencia, CA, USA). Anti-active caspase-3 was purchased from Promega (Madison, WI, USA). FITC-conjugated secondary antibody for immunohistochemistry was from Jackson ImmunoResearch (West Grove, PA, USA). ECL, Percoll, and secondary antibodies for immunoblotting were purchased from GE Life Sciences (Piscataway, NJ, USA). V5-OGC plasmid was a generous gift from Dr. Lash, Wayne State University (Detroit, MI, USA). The Bcl-2 plasmid was a generous gift from Dr. Hardwick, Johns Hopkins University (Baltimore, MD, USA).

CGN culture

CGNs were isolated from P7 Sprague–Dawley rat pups and cultured as previously described [24]. All animal manipulations were performed in accordance with and under the approval of the University of Denver Institutional Animal Care and Use Committee.

Immunohistochemistry

CGNs or CHO cells were transfected or treated as described in the figure legends and fixed with 4% paraformaldehyde. Next, the cells were blocked and permeabilized in PBS, pH 7.4, with 5% bovine serum albumin (BSA) and 0.2% Triton X-100 for 1 h, followed by incubation with the primary antibody overnight at 4 °C diluted in 0.2% Triton X-100 and 2% BSA in PBS. After this, the primary antibody was removed and the cells were washed 5× with PBS over 30 min. Next, the cells were incubated with secondary antibody and DAPI, diluted in 0.2% Triton X-100 and 2% BSA in PBS, for 1 h at room temperature. The cells were washed 5× with PBS over 30 min and placed in Anti-quench. Images were captured using a Zeiss Axioplan 2 fluorescence microscope equipped with a Cooke Sensicam CCD camera and Slidebook Image analysis software (Intelligent Imaging Innovations, Denver, CO, USA).