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## Letter to the Editor

# BI-1 protects cells from oxygen glucose deprivation by reducing the calcium content of the endoplasmic reticulum

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*Dear Editor*,

The yin and yang of life and death is regulated to a significant degree by members of the Bcl-2 family, which regulate the execution of apoptosis, the uniform cell death program that is essential for normal development and homeostasis of a multicellular organism.[1] The Bcl-2 family consists of proapoptotic members (e.g. Bax and Bak) that carry out the death program and antiapoptotic proteins counteracting it. Two protective family members, Bcl-2 and Bcl-XL were found previously to be increased by ischemic preconditioning (IP) of neuronal tissue,[2] meaning that their expression is induced by a short episode of ischemia followed by reperfusion, a treatment that induces tolerance against an otherwise lethal ischemia. Suppression of Bcl-2 by antisense oligodeoxynucleotides blocks the induction of tolerance making it a major determinant of IP.[3] We recently identified the antiapoptotic protein Bax inhibitor-1 (BI-1) to be about six-fold increased in preconditioned primary neuronal cultures (manuscript in preparation), which is of particular interest as BI-1 seems to exert its effect in cooperation with Bcl-2, with which it can be crosslinked and coimmunoprecipitated.[4] Xu and Reed[4] isolated BI-1 by screening clones in yeast for their ability to convey resistance against Bax-induced cell death and showed that BI-1 can protect mammalian cells from apoptotic stimuli like Bax, etoposide, staurosporine and serum-deprivation. BI-1 consists of six- to seven-transmembrane domains and it is not known how it protects from apoptosis.

Transient overexpression of EGFP-tagged BI-1 in CHO cells resulted in a prominent fluorescence with a reticular appearance in the cytosol and staining of the perinuclear envelope as previously described. Colocalization studies using antibodies against Bcl-2, the ER marker protein PDI (Protein Disulphide Isomerase) and the mitochondrial oxidative phosphorylation complex IV-I indicated that BI-1 mainly colocalizes (i) with Bcl-2 in the cytosolic compartment, but not in the perinuclear envelope, (ii) with PDI, (iii) not with the mitochondrial marker protein. (Figure 1a). From this, we concluded that BI-1 is expressed predominantly in the ER. Bcl-2's antiapoptotic mechanism, although not yet fully understood, seems to involve, at least in the ER, an alteration of ionic homeostasis, which is generally attributed to a possible function as an ion channel demonstrated in artificial lipid bilayers and also supported by its three-dimensional structure.[5] BI-1, with its six-transmembrane topology also resembles an ion channel, raising the intriguing possibility that BI-1 could participate in or even underlie the changes in calcium homeostasis ascribed to Bcl-2.

We transfected BI-1-EGFP into CHO cells loaded with the ratiometric calcium dye Fura-2 and measured cytosolic calcium induced by ATP in single cells. ATP activates Gq-coupled receptors leading to the production of inositol 1,4,5 trisphosphate (IP3), which then activates ER-localized IP3 receptors giving rise to an increase in cytosolic calcium. Transfected cells exhibited a dramatic decrease in ATP-mediated calcium transients. Most cells (72%) did not yield any calcium response at all; some displayed an attenuated (24%) and only 4% a normal rise in cytosolic calcium after stimulation (Figure 1b). Control cells transfected with EGFP alone yielded a normal calcium signal (not shown).

Execution of the apoptotic program includes a prolonged increase in mitochondrial calcium that leads to irreversible opening of the permeability transition pore and finally cell death. To answer whether BI-1 also attenuates the more important mitochondrial calcium, we measured BI-1 mediated changes in mitochondrial calcium with targeted aequorin constructs; aequorin is a protein isolated from jellyfish that emits light in the presence of calcium and its substrate coelenterazine. CHO cells stably expressing mitochondrially targeted aequorin[6] were transfected with BI-1, empty vector, or Bcl-2 as positive control,[7] and the calcium signal elicited with ATP and thapsigargin. Thapsigargin increases intracellular calcium by inhibition of the ER-specific calcium ATPase SERCA, which leads to a passive calcium shift out of the ER and into the cytoplasm, respectively mitochondria. BI-1 overexpression dramatically reduced the calcium increase in mitochondria elicited by both stimuli more prominently than Bcl-2 (Figure 1b). BI-1 overexpression attenuated ATP-mediated calcium rises by approximately 27% ($73.0 \pm 2.4\%$ of mock controls; $P < 0.005$; $n = 7$) and Bcl-2 by 12.5% ($87.5 \pm 2.2\%$ of mock controls; $P < 0.05$; $n = 7$).

Either BI-1 abates the absolute amount of calcium stored in the ER or it hampers with the efflux after induction. To solve this question, we used ER-targeted genetically encoded fluorescent indicators for calcium based on green fluorescent protein variants and calmodulin. Cameleon YC3.3 exhibits a medium calcium affinity and is localized in the ER.[8] Overexpressing YC3.3 and BI-1 resulted in a significant decrease of ER calcium (Ratio 538/476 nm; BI-1 $1.11 \pm 0.007$ compared

to mock 1.26±0.009, $P<0.0001$, $n=32$), which was not evident with a mutant lacking a coiled-coil region necessary for the reduction of Bax-mediated cell death in yeast ($\Delta C$ 1.22±0.01 compared to mock 1.26±0.009, $P>0.05$, $n=32$). ER calcium was always measured before and after exposure to thapsigargin to prove that our method really measures ER calcium (mock 0.94±0.006, BI-1 0.90±0.002, $\Delta C$ 0.92±0.08). We reproduced the data obtained in CHO cells in cell lines of neuronal (mouse hippocampal HT22 cells; mock 1.80±0.02, BI-1 1.64±0.02, $P<0.0001$, $\Delta C$ 1.80±0.02, $P>0.05$) and glial (rat C6 glioma cells; mock 2.06±0.01, BI-1 1.90±0.01, $P<0.0001$, $\Delta C$ 2.02±0.02, $P>0.05$) origin, to argue against a cell-type-specific epiphenomenon. BI-1, but not the $\Delta C$ mutant decreased the ER calcium in all three cell lines. The BI-1 effect was most pronounced in C6 glioma cells; the discrepancies between the

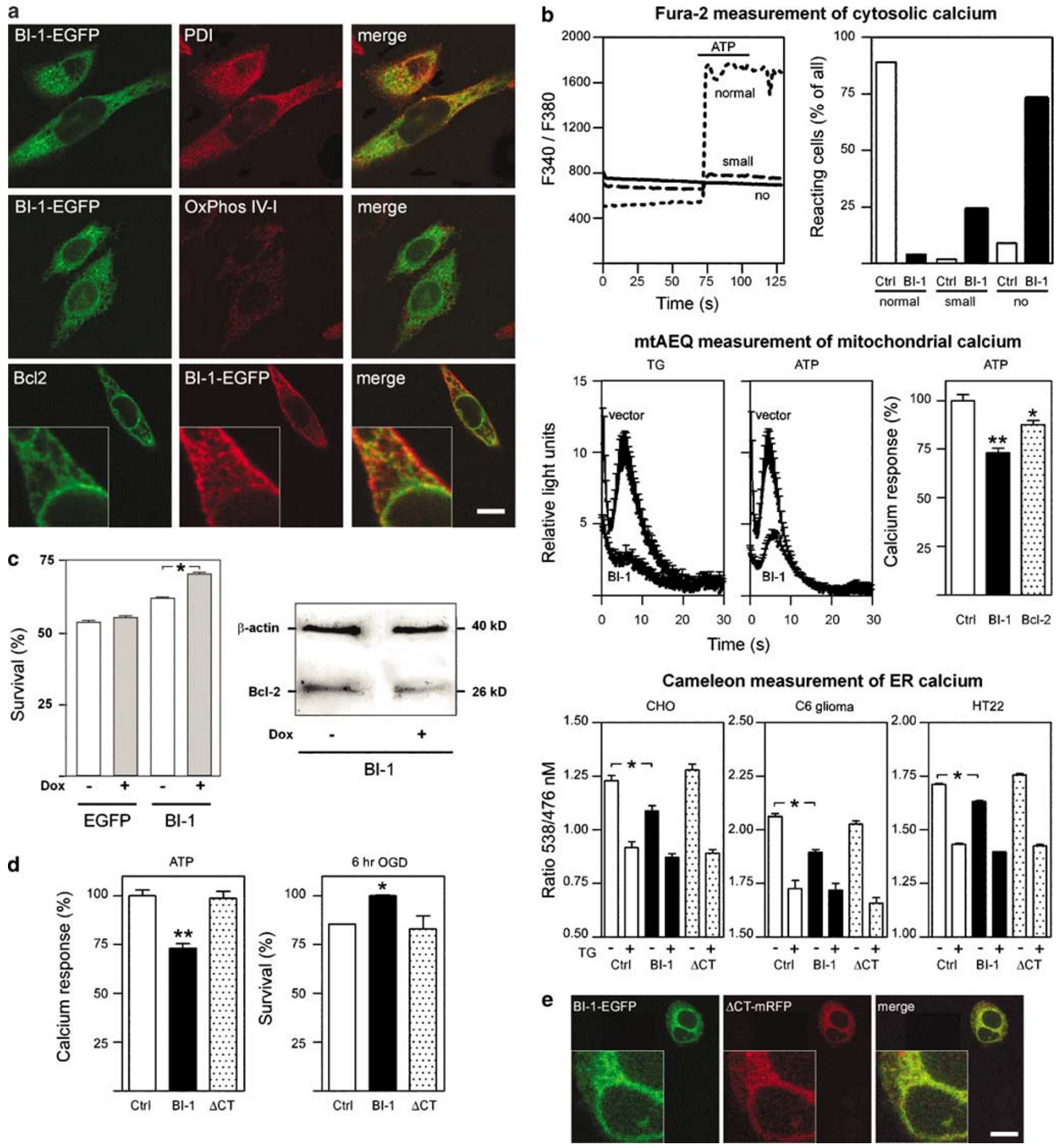


**Figure 1**

three cell lines probably reflect intrinsic differences in resting calcium levels.

Taken the very similar results of BI-1 and Bcl-2 overexpression, we wanted to exclude that the effect of BI-1 is mediated by an increase in Bcl-2 expression. Tetracycline protected inducible BI-1-, but not EGFP-expressing HEK-293 cells from oxygen glucose deprivation (OGD, BI-1+ dox 67.7±0.2%, BI-1− 60.7±0.1, EFGP+ 55.3±0.4, EGFP- 54.3±0.6, with a significant difference $P<0.05$ between BI-1+, and BI-1− and EGFP+, $n=4$, Figure 1c), but did not influence the expression of Bcl-2 as shown by Western blotting (Figure 1c). We then compared the ability of full-length BI-1 and the $\Delta C$ mutant to reduce the mitochondrial calcium rise induced by ATP and their ability to protect from OGD using the same cells. In our hands, inhibition of Bax-induced cell death, staurosporine, etoposide, and serum deprivation were less reproducible. The $\Delta C$ mutant was not able to protect CHO cells from 6 h OGD (mock 85.7±0.5% $P<0.05$ and $\Delta C$ 80.2±6.9%, N.S. of BI-1 mediated protection, $n=3$) and did not evoke the changes in mitochondrial calcium mobilization described above, suggesting that the two are functionally connected (Figure 1d) (BI-1 73.0±2.4%; $P<0.005$; $\Delta C$ 99.7±8.8% of mock controls, N.S.; $n=7$). Overexpression of full-length BI-1-EGFP and $\Delta C$-BI-1 C-terminally fused to monomeric red fluorescent protein mRFP1 showed complete coexpression of both constructs proving that the lack of function observed with the $\Delta C$ mutant is not due to changes in trafficking or ER localization (Figure 1e).

Our major finding is that BI-1 protects cells by reducing the ER calcium content, thereby attenuating ligand-induced and probably apoptosis-induced calcium transients in the cytosol and in mitochondria. This calcium phenotype is in principle similar to, but more pronounced than, the effect ascribed to Bcl-2.[7,9] The amount of releasable ER calcium is also reduced in cell lines lacking the buffer protein calreticulin, which similarly results in resistance against apoptosis.[10] Mouse embryonic fibroblasts lacking two proapoptotic proteins, Bax and Bak, are also resistant to a variety of apoptotic stimuli and have a much reduced ER calcium concentration.[11] Evidently, a reduction in ER calcium leads to cell survival, irrespective of the mechanism involved, probably by lowering the mitochondrial calcium accumulation that leads to irreversible opening of the permeability transition pore.

It is possible that BI-1 regulates the number or activity of ER calcium channels like the IP3 receptor, which is fostered by the observation that cells deficient in IP3 receptor type 1 are resistant to a variety of apoptotic stimuli.[12] However, as BI-1 possesses channel-like structural features one could hypothesize that it constitutes a homomeric channel itself that enhances the calcium permeability of the ER resulting in a leaky ER. Alternatively, it could form a heteromeric channel together with Bcl-2 or Bcl-XL that have channel-like properties.

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**Figure 1** (**a**) BI-1 colocalizes with Bcl-2 only in the ER. Confocal microscopy of CHO-mtAEQ cells transfected with 10 $\mu$g BI-1-EGFP (gift from John C Reed) stained with antibodies against Bcl-2 (polyclonal rabbit, Santa Cruz 1 : 100), the ER marker protein PDI (monoclonal, StressGen 1 : 200), and the mitochondrial protein Oxidative Phosphorylation Complex IV-I (monoclonal, Molecular Probes, 1 : 20). Secondary antibodies were a Cy3-conjugated goat anti-rabbit IgG (Amersham, 1 : 2000), an Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes, USA, 1 : 2000), and a Cy3-conjugated goat anti-mouse IgG (Santa Cruz, 1 : 1000). Scale bar represents 10 $\mu$m. Insets are magnified to 10 $\mu$m. (**b**) BI-1 attenuates agonist-induced cytosolic and mitochondrial calcium by reducing the ER calcium content. Cytosolic calcium traces in BI-1 transfected cells after stimulation with 100 $\mu$M ATP. Calcium changes imaged as ratio of fura-2 fluorescence (340 nm/380 nm) in BI-1-EGFP expressing CHO cells transfected with 5 $\mu$g BI-1-EGFP. Calcium responses of five experiments were grouped into no, small or normal response. Bar graphs show percentage of reacting cells according to the response with $n=48$ reacting cells. Mitochondrial calcium measured at 469 nm in CHO cells (transfected with 10 $\mu$g BI-1 or Bcl-2) stably expressing mitochondrially targeted aequorin (5 $\mu$M coelenterazine) after the addition of 100 $\mu$M ATP or 0.5 $\mu$M thapsigargin (TG). Traces show the mean of eight replicates ±S.E.M. Bar graphs show pooled data normalized to mock-transfected controls from seven experiments. ER calcium measured by ratiometric analysis of fluorescence (538 nm/476 nm) of CHO-mtAEQ, C6 glioma, and HT22 cells transfected with 1.5 $\mu$g cameleon YC3.3 and vector, BI-1, or $\Delta C$-BI-1 after excitation at 432 nm. Measurements were repeated after 5 min treatment with 0.5 $\mu$M TG. Bar graphs represent the mean of 32 replicates. Mann–Whitney test with $^*P<0.01$, $^{**}P<0.005$. (**c**) BI-1 protects from OGD without increasing Bcl-2 expression. HEK293 cells stably overexpressing inducible EGFP or BI- were treated with 1 $\mu$g/ml doxycycline for 24 h before a 2-h OGD. Viability was determined by MTT assays normalized to control cultures (mean±S.E.M., $n=4$ in triplicates). Western blots from the same cells probed with mixed antisera against Bcl-2 (1 : 200) and $\beta$-actin (rabbit polyclonal, Sigma 1 : 1000). Equal amounts of protein were applied per lane. Size is indicated. (**d**) Cell survival depends on BI-1's ability to reduce the mitochondrial calcium increase. Luminescent response measured at 469 nm in CHO-mtAEQ cells transfected with 10 $\mu$g BI-1 or $\Delta C$-BI-1 lacking the C-terminal domain ($\Delta$CT) after the addition of 100 $\mu$M ATP. Bar graphs show data from seven experiments normalized to mock-transfected controls (Ctrl). The same cells exposed to 6 h OGD with viability normalized to BI-1 and expressed in percent (mean±S.E.M., $n=4$). Mann–Whitney test with $^*P<0.05$, $^{**}P<0.005$. (**e**) The nonfunctional BI-1 mutant $\Delta$CT is also expressed in the ER. Confocal microscopy of CHO cells transfected with a total of 10 $\mu$g BI-1-EGFP and $\Delta C$-BI-1-mRFP1. Scale bar represents 10 $\mu$m. Insets are magnified to 10 $\mu$m