Decrease in Intracellular Glutathione Level Alters Expressions of B-cell CLL/Lymphoma 2 Family Members in the Mouse Retina

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Oxidative stress affects all intracellular macromolecules, and leads cells to death under unfavorable conditions. Glutathione (GSH) is known to play a critical role in the cellular defense against unregulated oxidative stress in mammalian cells including neurons. We previously demonstrated that GSH depletion induces cell death in the retina, but the mechanism of cell defense by GSH is still unclear. Thus, we here examined the effect of GSH depletion on expression of members of B-cell CLL/lymphoma 2 (Bcl-2) family (*bcl*-2, *bcl*-X_L, *bax*, *bak, n-bak* and *bad*) known to play key roles in determining cell viability. In order to deplete intracellular GSH, we systemically administrated buthionine sulphoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase to mice. After 0, 1, 4, and 7 days of BSO injection, total RNAs from retina of each animals were isolated and subjected to real-time reverse transcription (RT)-polymerase chain reaction (PCR) analysis. Expression of *bcl-*X_L increased one day after BSO injection, but was back to the basal level on day 4. Its expression decreased on day 7. Expressions of *bcl*-2 and *bax* were significantly decreased from 4 days after BSO injection, whereas expression of *bad* was not changed. An anti-apoptotic molecule, *bak* displayed a significant decrease 7 days after BSO injection, whereas neuronal cell specific Bak (Bcl-2 homologous antagonist/killer), N-Bak was not altered in its gene expression. Taken together, we demonstrated that decrease in intracellular glutathione level altered expressions of Bcl-2 family members in distinct manners. Our study implies a defensive mechanism of GSH against oxidative stress for retinal neuronal survival which may involve alteration of expression of Bcl-2 family members, especially Bcl-2 and Bcl-X_L. Thus, over-expression of Bcl-2 and Bcl-X_L may not the only but a critical factor in GSH-dependent cellular protection, and which implies Bcl-2 and Bcl-X_L may provide a potent therapeutic tool for cures against oxidative stress induced retinal degenerative diseases such as glaucoma, retinopathy, and age-related macular degeneration.

Key words —— oxidative stress, retina, glutathione, buthionine sulphoximine, B-cell CLL/lymphoma 2

INTRODUCTION

As mammals utilize oxygen, reactive oxygen species (ROS) is also elevated in cells. ROS are ubiquitous and has been implicated in various cellular processes such as signal transduction, immune defense, gene expression, and programmed cell death.[1)] Similarly to other systems, ROS are involved in various neuronal processes including the maintenance of neuronal functions and neuronal cell death.[2–5)] Under unfavorable conditions, ROS could accumulate damaging oxidative stresses to neurons, affect intracellular macromolecules, and in turn lead central nervous system (CNS) neurons to death.[1)]

Glutathione (GSH; L-γ-glutamyl-L-cysteinylglycine) is a predominant thiol in mammalian cells,[6, 7)] and has been mostly implicated as an anti-oxidant, in addition GSH is

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involed in the regulation of apoptosis.[8] Hence, GSH depletion may result in deleterious effects to cells. Under various pathological conditions, GSH indeed decreases in the tissues including the retina *e.g.* diabetic retinopathy,[9] glaucoma[10] and photooxidative damage.[11] A large body of evidence indicates that mitochondria is a key participant in the commitment phase of apoptosis (see review[12]), in specific binding of B-cell CLL/lymphoma 2 (Bcl-2) family member(s) to mitochondria is known to play a critical role in apoptosis triggering (See reviews[13–18]). In the detection and response stage of apoptosis, members of Bcl-2 family act as context-specific sensors for cell damage and converge on the mitochondria to trigger their permeabilization, releasing cytochrome c into the cytosol. Once cytochrome c is released, series of proteolytic maturation of caspases are triggered that is ultimately responsible for apoptotic cell death (See reviews[13–18]).

The members of Bcl-2 family play both anti-apoptotic and pro-apoptotic roles in cells.[19] Anti-apoptotic members show high structural and functional homology with Bcl-2. Bcl-2 is a proto-oncogene, forming ion conductive channels in mitochondria to prevent the release of cytochrome c. Pro-apoptotic members are consisted of Bax-like death factor members and pro-apoptotic BH3-only members.[19] Of each pro-apoptotic members, Bax (Bcl-2 associated X protein) and BAD (Bcl-X_L/Bcl-2-associated death promoter) are mostly studied. Bax shows high homology to Bcl-2 and is able to heterodimerize with Bcl-2. Overexpression of Bax is reported to enhance apoptosis in the cells, whereas Bax-null mice present resistance against apoptosis.[20] Bax-involved cell death is known to be inhibited by the presence of Bcl-2 or Bcl-X_L.[21] BAD is phosphorylated by various protein kinases and protects cells from apoptosis by raising the threshold at which mitochondria release cytochrome c to induce cell death.[22,23] When dephosphorylated, BAD is translocated to the mitochondria and interact with Bcl-2, and which facilitates the release of cytochrome c. Another death-promoting member is Bak (Bcl-2 homologous antagonist/killer) which shares structural and functional similarity to Bax.[19] Its pro-apoptotic effects are known to be regulated by Bcl-X_L.[24] A neuron-specific splicing variant of Bak, N-Bak has been also identified.[25] N-Bak is expressed exclusively in central and peripheral neurons, whereas Bak is expressed ubiquitously except in the neurons. Although N-Bak is implicated in neuronal survival, it is still able to induce apoptosis in non-neuronal cells.[26]

Retina is a part of the CNS, and perceives visual information. Unlike other parts of the CNS, the retina is destined to expose to high levels of oxidative stress cause of perception of environmental information in the form of light. Thus, the retina may contain a protection system from uncontrolled ROS elevation,[27] and which processes may involve expression of Bcl-2 family members. Here, we examined the expression of Bcl-2 family members in the mouse retina exposed to uncontrolled ROS production after GSH production was inhibited by buthionine sulfoximine (BSO), a γ-glutamylcysteine synthetase inhibitor.[28]

MATERIALS AND METHODS

Animals —— Male adult C-57BL mice (6–8 week old) were obtained from Hyochang Animal Inc. (Daejon, ROK). All experimental protocols were approved by Kyungpook National University Institutional Animal Care and Use Committees, and all applicable guidelines from the National Institute of Health Guide for the care and use of laboratory animals were followed.

Administration of BSO and Preparation of Retina RNA Samples —— Mice were intraperitoneally injected with BSO at 1.5 μg/kg. For controls, mice were injected with phosphate buffered saline (PBS pH 7.4). Five animals were used for each condition. After 0, 1, 4, and 7 days of injection, the animals were killed under anesthesia. The retina tissues were removed, and its total cellular RNA was purified using Trizol reagent (Gibco, Carlsbad, CA, USA).

Reverse Transcription (RT) and Real-time Polymerase Chain Reaction (PCR) Assay —— 5 μg of RNA from each source was reverse transcribed using oligo dT primers, random primers and reverse transcriptase AMV (Qiagen, Valencia, CA, U.S.A.). PCR was done in a 1/20 aliquot of the RT mixture for 40 cycles at 95°C for 10 sec, 57°C for 15 sec, and 72°C for 30 sec by using Taq DNA polymerase (Qiagen), or adjusted as needed. Real-time monitoring of PCR was done using Rotor-Gene3000 real-time thermal cycler (Corbett Life Science, Sydney, Australia). Primer pairs used for the analysis were summarized in Table 1.

Table 1. Primers and Expected Sizes of PCR Products with Each Primer Pair

Gene	Gene ID		Primer	Expected product size
Bcl-2	NM009741	sense	ctggcatcttctccttccag	183
		antisense	gacggtagcgacgagagaag	
Bcl-X_L	NM009742	sense	cgctacgacacagagttcca	153
		antisense	tccatctggcgatgtaatga	
Bax	NM007527	sense	tgcagaggatgattgctgac	173
		antisense	gatcagctcgggcactttag	
Bak	NM007523	sense	cgctacgacacagagttcca	250
		antisense	tccatctggcgatgtaatga	
N-Bak	AF402617	sense	cgctacgacacagagttcca	176
		antisense	tccatctggcgatgtaatga	
BAD	NM007522	sense	agggatggaggaggagctta	212
		antisense	cccaccaggactggataatg	
cyclophilin A	NM008907	sense	gtctccttcgagctgtttgc	235
		antisense	gatgccaggacctgtatgct	

RESULTS

Glutathione is known to play a critical role in the cellular defense against oxidative stress in mammalian cells, and GSH depletion affects vulnerability of cells to oxidant attack. D,L-buthionine sulfoximine (BSO) inhibits γ-glutamylcysteine synthetases,[28] as a result BSO causes decrease of intracellular GSH and induces uncontrolled oxidative stress in mammalian cells.[29] We have shown that systemic administration of BSO altered intracellular GSH level resulting in apoptotic neuronal cell death in the retina.[30] Moreover, the alteration of intracellular GSH level affected the expression of cyto-protective molecules, heat shock proteins.[31] Here, we determined if GSH depletion affects expression of Bcl-2 family members which are closely related to cell viability. We tested six Bcl-2 family members, Bcl-2, Bcl-X_L, Bax, BAD, Bak and N-Bak. Bcl-2 and Bcl-X_L play an anti-apoptotic role, whereas Bax, BAD and Bak are known to play a pro-apoptotic role.[19] N-Bak is a neuron-specific isoform of Bak,[25] having anti-apoptotic effects exclusively in neurons.[26] We first determined if these Bcl-2 family members are expressed in normal mouse retina. Total cellular RNA in the retina was isolated and subjected to reverse transcription and PCR analysis. We designed primers for real time PCR using Primer3 (http://fokker.wi.mit.edu/primer3/).[32] Each PCR product for Bcl-2 family members showed a single product with the molecular size as expected (Fig. 1).

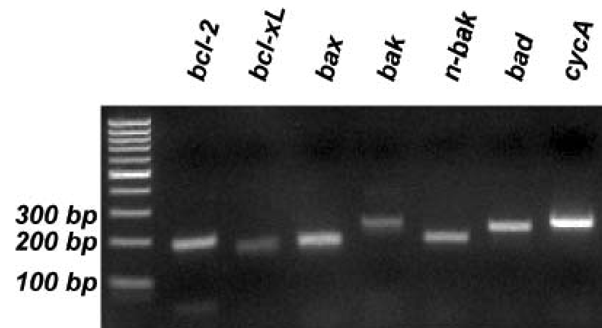

Fig. 1. Typical PCR Products of Bcl-2 Family Members under Our Experimental Condition

Molecular size standards (in base pairs) were also loaded.

We next examined expression of Bcl-2 family members after BSO administration, using real time RT-PCR analysis. Mice were injected with BSO, and sacrificed on day 0, 1, 4 and 7. Its total cellular RNA from the retina was isolated, and subjected to real-time RT-RCR analysis. At each time point, five mice were utilized. Expression of each Bcl-2 family members in the retina after BSO administration showed distinct patterns. An anti-apoptotic protein Bcl-2 showed a significant decrease in its gene expression on day 4 after BSO injection (Fig. 2A). The effect of BSO administration was maintained until 7 days. Another anti-apoptotic protein Bcl-X_L showed a significant increase in its gene expression on one day after BSO injection (Fig. 2B). The expression of *bcl-X_L* returned to the basal level on day 4, and displayed a significant decrease on day 7 after BSO injection. Pro-apoptotic protein Bax and Bak were gradually decreased in their gene expression from 4 days and 7 days after BSO injection respectively (Fig. 2C and 2E). Another pro-apoptotic protein BAD showed no significant expression change

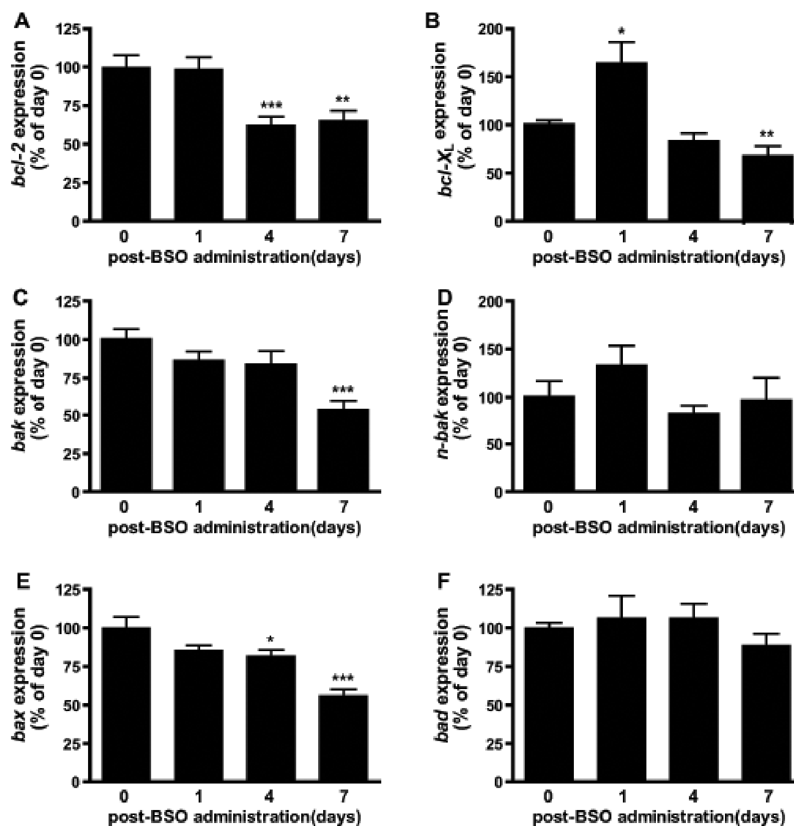

Fig. 2. Effect of Glutathione Depletion in Expression of Bcl-2 Family Members in the Mouse Retina

A-F show time-course of Bcl-2 family member expression in the retina after BSO administration. Each member is indicated on the representing graph. Quantitative analysis of Bcl-2 family member expression was performed after real-time PCR analysis. Data represents % of each genes expressed on day 0 in the retina of five different animals (mean ± standard error of the mean). Statistical significances are indicated (student *t*-test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

at its message level at least within 7 days after BSO injection (Fig. 2F). Neuron-specific isoform of Bak, N-Bak also did not show any significant changes in its gene expression (Fig. 2D).

DISCUSSION

We previously demonstrated that GSH decrease induced cell death in the retina, but the mechanism of the retinal cell death caused by GSH depletion has been still unknown.[30, 31] An anti-apoptotic molecule, Bcl-2 is reported to prevent the loss of photoreceptor cells and horizontal cells[33] or delay photoreceptor cell degeneration[34] in retinal degenerative disease animal models, and to be involved in regeneration[35] and long-term survival[36] of lesioned retinal axons. In addition, *bcl*-2 null mice showed loss of retinal ganglion cells.[37] Thus, Bcl-2 appears to play a critical role in retinal cell survival. Our results indicate that Bcl-2 seems to be involved in retinal cell survival against unregulated oxidative stress, in fact Bcl-2 has been reported to protect the GT1-7 neural cell line against BSO-induced cell death.[38] We previously showed the peak of cell death by GSH depletion reached 4 day after BSO injection.[30] We found the expression of *bcl*-2 significantly decreased after 4 days of BSO administration, implicating a role of Bcl-2 in retinal cell survival under oxidative stress. Of our interest, the expression of *bcl*-2 was not fully recovered on day7 when the retinal cell death no longer increased,[30] suggesting that Bcl-2 may not be involved in establishment of new equilibrium of cell homeostasis in the retina after unregulated oxidative stress. Taken together, Bcl-2 may play a critical role in retinal cell survival under oxidative stress, but does not seem to be the only factor playing such role.

Another well characterized Bcl-2 family member, Bcl-X_L significantly increased after BSO administration. Bcl-X_L is known to promote cell survival by regulating the cytochrome c release into cytosol or binding to Apaf-1 to prevent caspase-9 activation.[39] The early resistance of the retinal cells against GSH depletion[30] may come from the upregulation of Bcl-X_L. The decrease in the gene ex-

pression of both anti-apoptotic proteins Bcl-2 and Bcl-X_L may permit the retinal cells to be exposed to unregulated oxidative stress without appropriate defense, and which may result in apoptosis of the retinal cells afterward. Similarly to the case of Bcl-2, Bcl-X_L does not appear to be the only factor regulating retinal cell survival.

Expression of *bad* was not altered by GSH depletion in the retina, suggesting that BAD may not be involved in oxidative stress induced cell death in the retina. Or post-translational modification of BAD without changes in its expression level plays a role in regulation of the oxidative stress induced cell death in the retina, since dephosphorylation of BAD induces translocation to the mitochondria and interaction with Bcl-2, and which in turn trigger apoptosis.[22, 23]

Bax is a multidomain pro-apoptotic member of Bcl-2 family, and large body of reports clearly indicates that Bax plays a pro-apoptotic role also in the nervous system.[19] Bax null mice exhibit long-term protection against various apoptotic stimuli,[40] resistance of postnatal retinal ganglion cells to axotomy,[37] and critical involvement in retinal developmental apoptosis.[41] Hence one may expect an increase in *bax* expression after GSH depletion which causes apoptosis in the retina. Very intriguingly, *bax* expression in the retina was decreased instead. At present, it is hard to explain why *bax* expression decreases under GSH depletion, but it has been reported that the ratio of either Bcl-2/Bax or Bcl-X_L/Bax is critical in determination of cellular viability. The ratio of Bcl-2/Bax is indeed altered in neurodegenerative diseases such as Alzheimer disease,[19] i.e. proportion of Bcl-2 expression decreases whereas that of Bax increases. In our study, expressions of both *bcl*-2 and *bax* decrease on day 4 of BSO treatment, but the expression of *bcl*-2 appeared to decrease more than that of *bax*, indicating that the ratio of Bcl-2/Bax may be shifted to the side of neurodegeneration, i.e. cell death in the retina (Fig. 3). On day 7 of BSO treatment, the ratio of Bcl-2/Bax is back to the basal level, and which is comparable with our previous observation that the retinal cell death was back to the basal level.[30] The ratio of Bcl-X_L/Bax significantly increased on day 1 of BSO administration, and which is also comparable with our previous observation that the retinal cell death was not altered in 24 hr of BSO injection.[30] Alternatively, Bax may play a pro-survival role in the retina as previously described in the nervous system.[42]

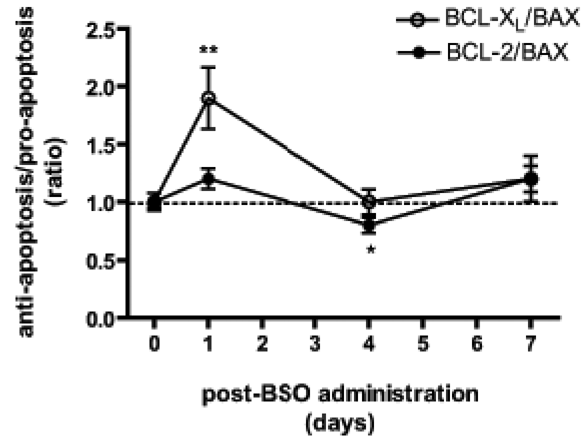

Fig. 3. Ratio of Bcl-2/Bax and Bcl-X_L/Bax

The ratio of either Bcl-2/Bax or Bcl-X_L/Bax is critical in determination of cellular vulnerability. Statistical significances are indicated (student *t*-test; $^{*}p < 0.05$, $^{**}p < 0.01$).

A death-promoting member, Bak was identified in the normal retina, although it is known to be expressed ubiquitously except in the neurons.[26] Bak may be expressed in Muller cells and be involved in neuronal survival. After 7 days of BSO administration, expression of Bak was significantly decreased. Bak is structurally and functionally similar to Bax,[19] and its pro-apoptotic effects are known to be regulated by Bcl-X_L.[24] In fact, these two Bcl-2 family members displayed similar changes in their expressions. Moreover, the ratio of Bcl-X_L/Bak also significantly increased on day 1 of BSO administration, and which is also comparable with the ratio of Bcl-X_L/Bax during the period of BSO administration (Fig. 3). Thus, these two molecules may play a similar role in retinal cell vulnerability under unregulated oxidative stress. Recently, a neuron specific splicing variant of Bak, N-Bak has been identified.[26] N-Bak plays an anti-apoptotic role solely in neurons,[26] but either expression or functions of N-Bak in the retina has not been revealed yet. Here, we first identified the expression of N-Bak in the retina. We failed to observe any alteration in the gene expression of N-Bak after GSH depletion, indicating that N-Bak may play the anti-apoptotic role in the retina against insults besides unregulated oxidative stress provoked by GSH depletion.

Our study is solely focused on changes in gene expression of Bcl-2 family members, thus several questions such as changes in protein expression should be still answered for our major conclusion. In spite of such limitations, our study contains intriguing and yet important observation on the effect of GSH decrease on Bcl-2 family genes, suggesting that the important cellular protection mechanisms

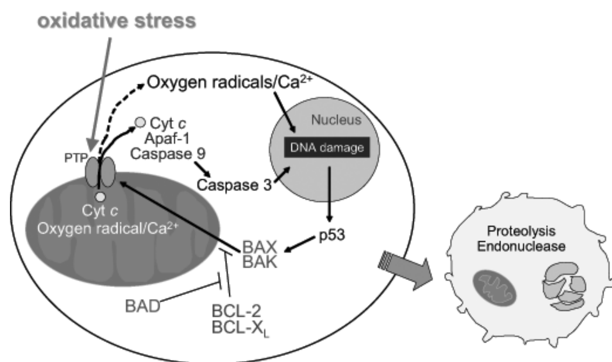

Fig. 4. Working Hypothesis

Bcl-2 family members interact each other to change the status of cell viability after exposure to oxidative stress.

depend on Bcl-2 family upon oxidative stress challenge. Further study including validation of gene expression using immunoblot analysis and determination of cell types showing most changes in Bcl-2 family members under unregulated oxidative stress using immunohistochemical analysis.

In summary, GSH depletion alters members of Bcl-2 family in the retina, and Bcl-2 and Bcl-X_L appear to play a critical role in retinal cell survival under unregulated oxidative stress (Fig. 4). Pro-apoptotic Bcl-2 family members, BAD, Bax and Bak showed different expression patterns at the message level. Our study implies a defensive mechanism of GSH against oxidative stress for retinal neuronal survival which may involve alteration of expression of Bcl-2 family members, especially Bcl-2 and Bcl-X_L. Thus, over-expression of Bcl-2 and/or Bcl-X_L may prevent the retinal cells from oxidative stress related retinal degenerative diseases such as glaucoma, retinopathy, and age-related macular degeneration.

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