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*J Immunol* 1998; 160:2590-2596; 
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A Physiologic Role of Bcl-xL Induced in Activated Macrophages

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Activated macrophages produce nitric oxide (NO) that is an important effector molecule for their antimicrobial and antitumor activities. Since this NO is also toxic for themselves, they have self-defense mechanisms. To elucidate the mechanisms in a physiologic condition, expression of bcl-2 family genes were examined in peritoneal macrophages and RAW264 macrophage cell line activated with IFN-γ and LPS. Bcl-xL, but not bcl-2 and bax mRNA, was highly inducible within 3 h after stimulation. The induction required new protein synthesis, but was independent of effects of synthesized NO. Since activated RAW264 were more resistant to NO-induced apoptosis mediated by the exposure to S-nitroso-N-acetyl-penicillamine (SNAP) than nonactivated RAW264, the inducible Bcl-xL may play a role in the protection from NO toxicity. To confirm the protective function, RAW264 were stably transfected with bcl-xL. Those transfecteds activated with IFN-γ and LPS appeared highly resistant to NO-induced cell death detected within 24 h after stimulation, although their NO production was similar to those of parental RAW264 and neonycin control-transfected cells. Furthermore, bcl-xL transfecteds displayed substantial protection from SNAP-induced apoptosis. These results establish a link between self-defense to the synthesized NO and the induction of Bcl-xL in activated macrophages.


Nitric oxide (NO) has been implicated in physiologic roles in immune effector mechanisms, intra- and intercellular communications, and neurotransmissions (1, 2). NO is produced from the amino acid arginine by the enzyme NO synthase (NOS). To date, just three mammalian NOS genes have been identified (3). Two of them are expressed constitutively in neurons (NOS I) or endothelium (NOS III). Expression of the third isofrom (iNOS, NOS II) is inducible in many types of cells, including activated macrophages, vascular smooth muscle cells, and hepatocytes (4, 5). Large amount of NO can be produced by iNOS in activated macrophages within several hours after inflammatory stimulations, and works in macrophage toxicity against tumors, intracellular pathogens, and extracellular organisms (5). This NO production continues in the macrophages for a few days and has been reported to induce apoptosis in some of the macrophages from 24 h after activation (6, 7). Sensitivity of cells to NO toxicity seems to be different among cell types. Pancreatic β cells (8, 9) and thymocytes (10) are quite susceptible to, but hepatocytes (11) and mesangial cells (12) are less susceptible to NO toxicity, suggesting the presence of protective mechanisms in the latter cells. Several defense mechanisms have been reported for the NO-induced apoptosis (13). Since activated macrophages may also have self-defense mechanisms. However, the mechanisms in a physiologic condition are not well known.

Bcl-2 family genes play a role in regulating apoptosis (14, 15) and may be a key molecule for self-defense mechanisms in activated macrophages. Bcl-2 and its relatives, bcl-xL and bax, encode intracellular membrane-bound proteins. Bcl-2 and Bcl-xL protein are functionally equivalent (16–18). They enhance the survival of several cell types and prevent apoptosis induced by a wide range of agents, including oxidative stress (16, 19, 20). However, expression of bcl-xL appears to be more restricted than that of bcl-2, and mitogenic activation of mature cells strongly stimulates expression of bcl-xL, but only modestly increases that of bcl-2 (21). These differences may reflect their functional roles in cell survival. On the other hand, Bax antagonizes the survival function of Bcl-2. Bax forms homodimers that comprise an active trigger for cell death. Bax also makes heterodimers with Bcl-2 and Bcl-xL (22) that prevent the formation of toxic Bax homodimers, resulting in survival. Therefore, the ratio of Bcl-2 or Bcl-xL to Bax appears to determine the fate of cells (14, 15, 22).

Expression of iNOS is strongly induced in RAW264.7 murine macrophage cell line activated with IFN-γ and LPS within 3 h after stimulation and remains elevated for a few days (5, 23). Continuous NO production generated by the iNOS gradually induces apoptosis in some of the RAW264.7 within 24 h after stimulation. This NO-mediated apoptosis can be blocked by overexpression of bcl-2 (23), suggesting that Bcl-2 might play a role in the self-defense mechanisms. However, the protective activity of Bcl-2 is not physiologic since the endogenous bcl-2 gene is not up-regulated in activated RAW264.7 (23). To elucidate the mechanisms in a physiologic condition, we analyzed expression of bcl-2 family genes in peritoneal macrophages and RAW264 macrophage cell line activated with IFN-γ and LPS. We show in this study that expression of bcl-xL was highly inducible in activated macrophages. We discuss the function of Bcl-xL as a physiologic molecule that plays a key role in the self-defense mechanisms against NO toxicity in macrophages.
Materials and Methods

Mice

C57BL/6CrSlc mice were purchased from Japan SLC Co. (Hamamatsu, Japan).

Reagents

Murine IFN-γ with sp. act. of 1 × 10^7 U/mg was purchased from Genzyme Corp. (Cambridge, MA). LPS (from Escherichia coli, serotype 0111: B4) and cycloheximide (CHM) were purchased from Sigma Chemical Co. (St. Louis, MO). NO inhibitor, L-N5-iminoethyl ornithine dihydrochloride (t-NIO) and NO generation agent, S-nitroso-N-acetyl-D,L-penicillamine (SNAP) were purchased from Cayman Chemical Co. (Ann Arbor, MI). IL-4 (10^4 U/ml) was prepared from culture supernatant of X63Ag8-653 cells transfected with murine IL-4 gene (24).

Macrophage cell culture

Macrophages from mice were prepared from resident peritoneal cells. Briefly, peritoneal cells were harvested without elicitation and cultured in six-well tissue culture plates for 2 h at 37°C in 5% CO₂. After nonadherent cells were removed by extensive washing, adherent cells were harvested by trypsinization and used as peritoneal macrophages. RAW264 murine macrophage cell line was obtained from RIKEN Cell Bank (Tsukuba, Japan). Macrophages were cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 100 µg/ml streptomycin sulfate (Wako Chemical Co., Osaka, Japan), 100 U/ml penicillin G potassium (Banyu Pharmaceutical Co., Tokyo, Japan), and 10% (v/v) heat-inactivated FCS (Bioserum, Victoria, Australia).

Assay for NO synthesis

Synthesis of NO by activated macrophages was measured by the assay for nitrite (NO₂⁻), a stable NO oxidation product, in culture supernatants, as previously described (25). Briefly, 100 µl of culture supernatants were mixed with an equal volume of Griess reagent (0.5% sulfanilamide, 0.05% N-1-naphthyl ethylenediamine dihydrochloride in 2.5% H₃PO₄) in a 96-well tissue culture plate for 10 min at room temperature. The absorbance of samples was measured on an ELISA plate reader (Bio-Rad, Richmond, CA) at 570 nm. The nitrite concentration was calculated using sodium nitrite as a standard.

Northern blot analysis

Total RNA was isolated from cultured macrophages using the TriZOL RNA isolation reagent (Life Technologies). Total RNA (5–10 µg) was loaded on a 1% agarose gel in MOPS buffer containing 6% formaldehyde, transferred to a nylon membrane (Boehringer Mannheim GmbH, Mannheim, Germany), and fixed by cross-linking with UV irradiation and by baking at 80°C for 3 h. The filter was hybridized with digoxigenin-labeled probe overnight at 50°C. Following hybridization, the filter was washed twice with 0.1× SSC and 0.1% SDS at 55°C for 15 min. The probe on the filter was detected with sheep anti-digoxigenin Abs conjugated with alkaline phosphatase. The Ab detection reaction was performed using an enhanced chemiluminescent detection system (Boehringer Mannheim). Full-length murine iNOS cDNA (26) (a gift from Dr. K. Nishina, Kobe University, Kobe, Japan), murine bcl-2 cDNA (27), murine bax cDNA (22), and rat bcl-xL cDNA (26) (a gift from Dr. S. Ohta, Nippon Medical School, Kawasaki, Japan) were subcloned into pGEM vectors and labeled by digoxigenin, using PCR with T7 and SP6 primers, then used as a probe. Amounts of the mRNAs were measured by the densitometer (AE-6920-MF; Atto, Tokyo, Japan).

Transfection of bcl-xL cDNA into a RAW264 cell line

The bcl-xL cDNA was subcloned into an XbaI site of the plasmid pEF-BOS, which harbors an elongation factor 1e promoter (29). The pEF-BOS/bcl-xL (20 µg) was transfected into RAW264 with 1 µg of the pST-neoB, which carries a neomycin-resistant gene (30). Transfection was performed by electroporation with a Gene Pulser (Bio-Rad) at 0.35 kV in a 0.4-cm cell. After selection with 0.5 mg/ml geneticin G418 (Life Technologies), five clones that stably expressed the exogenous bcl-xL gene were established. The clone 35-1 with the highest level and the clone 35-3 with the lowest level of bcl-xL mRNA among five transfectants (data not shown) were used for following experiments. Parental RAW264 as well as two clones of pST-neoB-transfectants (neo-1 and neo-2) were used as controls. Cell morphology, proliferative behavior, and expression of bcl-2 and bax mRNA were comparable between bcl-xL transfectants and parental RAW264 (data not shown).

Expression of iNOS and bcl-2 family gene expressions in macrophages stimulated with IFN-γ and LPS. Peritoneal macrophages (A) and RAW264 (B) were stimulated with IFN-γ (40 U/ml) and LPS (10 µg/ml). Total RNA was isolated from the macrophages at the indicated times after stimulation. Levels of iNOS and bcl-2 family gene (bcl-2, bcl-xL, and bax) mRNAs in total RNA were analyzed by Northern blot. WEHI 231 that expresses bcl-2, but not bcl-xL (20), was used as a positive control for bcl-2 mRNA.

FACS analysis for apoptotic cell death

Cell viability was examined by the propidium iodide (PI) exclusion method, as described (31). Briefly, cultured macrophages were harvested by trypsinization and resuspended in staining buffer (0.1% sodium azide, 3% FCS in PBS) with 2 µg/ml PI. PI uptake in each cell was analyzed on FACS Calibur (Becton Dickinson, Mountain View, CA) using Cell Quest software. Data were displayed as percentages of PI-stained cells.

Since the nuclei in apoptotic cells show a uniform reduction in DNA stability with PI, which is indicated by the appearance of a subdiploid fraction of cells on the DNA histogram (32), FACS analysis of PI-stained nuclei was performed to detect apoptotic cells. Briefly, harvested macrophages were incubated in hypotonic lysing buffer (0.1% sodium citrate, 0.01% Triton X-100, and 0.1 mg/ml PI) at 4°C for 4 h. DNA content in each cell was analyzed on FACS Calibur using Cell Quest software for Macintosh. Data were displayed as percentages of apoptotic (hypodiploid) nuclei.

Results

Bcl-xL mRNA is up-regulated in peritoneal macrophages and RAW264 macrophage cell line stimulated with IFN-γ and LPS

Expression of iNOS and bcl-2 family genes (bcl-2, bcl-xL, and bax) was analyzed in peritoneal macrophages stimulated with IFN-γ and LPS by Northern blot (Fig. 1A). Expression of iNOS was induced within 3 h and reached to the maximum at 6 h after stimulation. Bcl-xL mRNA was faintly detectable before stimulation. The expression was up-regulated within 3 h, reached the plateau level (5.2-fold increase) from 6 h, and was sustained for at
least 24 h after stimulation. Bcl-2 mRNA was detected before stimulation. However, it was not up-regulated at all. Bax mRNA was observed at relatively high levels before stimulation and up-regulated (1.8-fold) from 6 h after stimulation.

When RAW264 were stimulated with IFN-γ and LPS, the similar kinetics of those gene expressions was observed (Fig. 1B). RAW264 without stimulation did not express a detectable level of iNOS mRNA. Expression of iNOS was induced from 3 h after stimulation. Bcl-xL was rapidly up-regulated within 1 h and maintained the high level (26-fold increased) until at least 48 h after stimulation. As is different from peritoneal macrophages, bcl-2 expression was not observed at all. Bax was also detected before stimulation, up-regulated (two-fold) from 12 h after stimulation, and maintained until 48 h after stimulation.

The up-regulation of bcl-xL mRNA in RAW264 stimulated with IFN-γ and LPS is not controlled by synthesized NO

Expression of iNOS and bcl-xL was induced simultaneously in activated macrophages, suggesting that their induction mechanisms are very similar. The similarity was further analyzed in RAW264 stimulated with IFN-γ or LPS (Fig. 2). Expression of iNOS and bcl-xL was induced in RAW264 stimulated with LPS in a dose-dependent manner. Those expressions were also induced by stimulation of IFN-γ alone. When RAW264 were costimulated with IFN-γ (40 U/ml) and LPS (1 μg/ml), expression of iNOS was synergistically up-regulated. When the amount of iNOS mRNA in RAW264 activated with IFN-γ was arbitrary 1, the relative amounts in RAW264 stimulated with LPS or with IFN-γ and LPS were 3.5 or 13. However, the synergistic response such as the iNOS expression was not obvious in expression of bcl-xL, since the relative amounts of bcl-xL mRNA in the cells activated with LPS or with IFN-γ and LPS were 3.7 or 4.6.

As a large amount of NO synthesized by the induced iNOS might inhibit the synergistic up-regulation of bcl-xL in RAW264 activated with IFN-γ and LPS, the up-regulation of bcl-xL was examined in activated RAW264 without NO generation. i-NIO, l-arginine analogue, is a potent, fast acting, and irreversible inhibitor of NO generation (33). When RAW264 were stimulated with IFN-γ and LPS in the presence of i-NIO, the up-regulation of bcl-xL mRNA was not affected (Fig. 3A), while NO2 production in culture supernatants was drastically repressed (Fig. 3B). This was repeated using another NO inhibitor, IL-4. IL-4 inhibits iNOS production at the transcriptional level (4, 34). When RAW264 were cultured with IL-4 for 1 h before stimulation with IFN-γ and LPS, both iNOS mRNA (Fig. 3A) and NO2 production (Fig. 3C) were significantly reduced in a dose-dependent fashion. However, the up-regulation of bcl-xL was not affected (Fig. 3A). These data indicate that the up-regulation of bcl-xL is not controlled by synthesized NO.

The up-regulation of bcl-xL mRNA requires the de novo protein synthesis in RAW264 stimulated with IFN-γ and LPS

Since the bcl-xL mRNA was rapidly up-regulated in activated macrophages after stimulation (Fig. 1), expression of bcl-xL may be induced without new protein synthesis in the macrophages such as an immediate early gene (35). To examine the possibility, RAW264 were precultured with CHM, a reversible protein synthesis inhibitor (36), for 30 min and then stimulated with IFN-γ and LPS. Expression of c-fos mRNA (one of the immediate early genes) in the RAW264 was strongly up-regulated by the CHM treatment (data not shown). However, the CHM treatment blocked
the up-regulation of bcl-xL as well as that of iNOS (Fig. 4). Thus, both inductions required the de novo protein synthesis in RAW264 stimulated with IFN-γ and LPS.

**Bcl-xL blocks NO-induced cell death of activated macrophages**

To examine the activity of Bcl-xL in activated RAW264 to protect from NO toxicity, RAW264 were stimulated with IFN-γ and LPS for 2 h to up-regulate Bcl-xL and then exposed to NO donor, SNAP. Percentages of dead cells in the activated RAW264 were analyzed at 8 h after SNAP exposure by the PI exclusion with FACS. As shown in Figure 5, approximately 23% of nonactivated RAW264 were dead by the SNAP exposure, as expected (23). The pretreatment of RAW264 with IFN-γ and LPS clearly reduced the number of dead cells (7%), suggesting that the up-regulation of Bcl-xL protects RAW264 from NO-induced cell death.

To confirm the antiapoptotic effect of Bcl-xL in activated macrophages, bcl-xL transfectants (35-1 and 35-3; see Materials and Methods) were stimulated with IFN-γ (40 U/ml) and LPS (10 μg/ml) for 24 h and then further cultured in the presence of 2 mM SNAP for 8 h. Percentages of dead cells in the RAW264 were examined by the PI exclusion method with FACS. Data represent the mean ± SD from three independent experiments.

To examine the activity of Bcl-xL in activated RAW264 to protect from NO toxicity, RAW264 were pretreated with CHM (3 μg/ml) for 30 min and then stimulated with IFN-γ (40 U/ml) and LPS (10 μg/ml) for 4 h. Levels of iNOS and bcl-xL mRNA in total RNA were analyzed by Northern blot.

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**FIGURE 4.** Effects of CHM on the up-regulation of bcl-xL in RAW264 stimulated with IFN-γ and LPS. RAW264 were pretreated with CHM (3 μg/ml) for 30 min and then stimulated with IFN-γ (40 U/ml) and LPS (10 μg/ml) for 4 h. Levels of iNOS and bcl-xL mRNA in total RNA were analyzed by Northern blot.

**FIGURE 5.** Protective activity of Bcl-xL in RAW264 to SNAP-induced cell death. RAW264 were stimulated with IFN-γ (40 U/ml) and LPS (10 μg/ml) for 2 h and then further cultured in the presence of 2 mM SNAP for 8 h. Percentages of dead cells in the RAW264 were examined by the PI exclusion method with FACS. Data represent the mean ± SD from three independent experiments.

**FIGURE 6.** Protective activity of Bcl-xL in RAW264 to NO-induced cell death. Bcl-xL transfectants (35-1 and 35-3), parental RAW264, and neomycin transfectants (neo-1 and neo-2) were stimulated with IFN-γ (40 U/ml) and LPS (10 μg/ml) for 24 h in the presence (open bar) or absence (closed bar) of l-NIO (100 μM). A, Percentages of dead cells in the RAW264 were analyzed by the PI exclusion method with FACS. B, NO synthesis by those transfectants was analyzed by measuring NO2 concentration in the culture supernatants. Values are expressed as μM NO2/106 cells/24 h and represent the mean ± SD (n = 3 per group). C, Apoptotic subdiploid cells in the RAW264 were detected by PI staining with FACS.
examined by PI staining with FACS. The overexpression of Bcl-xL partially protected apoptotic cell death of activated RAW264 (Fig. 6C), despite a concomitant production of NO$_2$ (Fig. 6B). Although the exogenous Bcl-xL protected RAW264 from NO-induced cell death, the Bcl-xL did not influence the level of NO produced by the RAW264 (Fig. 6B).

Since the apoptotic cell death was induced in some of nonactivated RAW264 within 8 h after SNAP exposure (Fig. 5), the protective activity of exogenous Bcl-xL in RAW264 was examined to the SNAP-induced apoptosis. Figure 7A shows that 28% of parental RAW264 were dead at 8 h after SNAP exposure. Similar results were obtained from neo-1 and neo-2. In contrast, 35-1 and 35-3 displayed better survival. Percentages of apoptotic cells in 35-1 were substantially less than those in control (Fig. 7B). The 35-1 showed only 4.3% DNA fragmentation, whereas the parental RAW264 showed 23.5% at 8 h, and 16.7 and 43.6% at 24 h after SNAP exposure, respectively.

**FIGURE 7.** Protective activity of exogenous Bcl-xL in RAW264 to SNAP-induced cell death. Bcl-xL transfectants (35-1 and 35-3), parental RAW264, and neomycin transfectants (neo-1 and neo-2) were cultured with 2 mM SNAP for 8 or 24 h. A. Percentages of dead cells in the RAW264 at 8 h after SNAP exposure were examined by the PI exclusion method with FACS. Data represent the mean ± SD from three independent experiments. B. Apoptotic subdiploid cells in the RAW264 were detected by PI staining with FACS.

**Discussion**

Bcl-xL, as well as Bcl-2, delay cell death following diverse apoptotic stimuli (17, 21, 28, 37, 38). However, the tissue distribution and expression level of bcl-xL are not identical to those of bcl-2. Those differences may reflect their physiologic roles in the regulation of apoptosis (14). In lymphocytes, bcl-xL and bcl-2 are expressed in nearly reciprocal patterns during development. Bcl-2 is highly expressed in long-lived lymphocytes such as single-positive medullary thymocytes, peripheral T cells, memory B cells, and their progenitors, and is critical for their survival (14, 39, 40). Bcl-xL is transiently expressed in immature intermediate cells such as pro- and pre-B cells and double-positive T cells that run the gauntlet of selection (41, 42). In macrophages, Bcl-xL, but not Bcl-2, was up-regulated by activation with IFN-γ and LPS (Fig. 1), although bcl-2 was slightly expressed in resting peritoneal macrophages (Fig. 1A). Since Bcl-xL transiently induced in differentiating lymphocytes plays a role in their selection processes (41, 42) and kinetics of the up-regulation in bcl-xL was coincident with that in iNOS (Fig. 1), Bcl-xL induced in activated macrophages may also play a role in their transient protection from NO-induced apoptosis.

Bcl-xL is a potent repressor of apoptosis induced by NO and reactive oxygen species (19), suggesting that Bcl-xL controls a common pathway for apoptosis mediated by NO and oxidants (16, 20). Bcl-xL homodimers have been suggested to form protective complexes against apoptosis. Since Bcl-xL can form heterodimers with several other proteins, including BAG-1, Bax, Bad, and Bcl-xS, its protective effects can be further enhanced by BAG-1 and diminished by Bax, Bad, and Bcl-xL (14, 15). Bax homodimers comprise an active trigger for cell death (15, 22). Expression of Bax was up-regulated (within twofold) in macrophages from 6 to 12 h after activation, and was not coincident with the induction in iNOS and bcl-xL (Fig. 1). Furthermore, the inducibility (5-fold in peritoneal macrophages and 26-fold in RAW264) of bcl-xL was much larger than that of Bax in activated macrophages until 12 h after stimulation. Therefore, amounts of Bcl-xL homodimers and Bcl-xL/Bax heterodimers may increase and that of Bax homodimers may decrease to result in the protection from NO-induced apoptosis. Since some of activated macrophages became apoptotic from 12 to 24 h after stimulation (Fig. 6A), this NO-induced self-destruction may be explained by the up-regulation of Bax in activated macrophages from 6 to 12 h after stimulation (Fig. 1).

NO is toxic for macrophages via several mechanisms, including inhibition of mitochondrial respiration and DNA synthesis, and initiation of DNA strand breaks (1, 3–5). In some instances, NO has been shown to enhance cellular oxidative injury (4). Several molecules other than Bcl-xL may be able to regulate susceptibility of macrophages against NO toxicity. Kim et al. demonstrated the existence of inducible cellular resistance mechanism by hsp32 (heme oxygenase) in hepatocytes against NO (11). Furthermore, many of the stimuli that induce iNOS expression are known to increase expression of metallothionein (43), and overexpression of metallothionein reduces the sensitivity to cell injury and DNA double strand break by NO in National Institutes of Health 3T3 cells (44). Thus, metallothionein may play a physiologic role in activated macrophages since normal macrophages can produce metallothionein (43). Heat shock as well as overexpression of a heat-shock protein (hsp70) also induce resistance to NO toxicity in islet cells (45). Indeed, an NO donor has been shown to induce...
hsp70 expression in a hepatoblastoma cell line and in various organs of animals (46). Moreover, pretreatment or repeated treatments of macrophages with nontoxic doses of IFN-γ and LPS increase expression of hsp70 and result in cell resistance to NO toxicity (47, 48). Since those IFN-γ and LPS treatments also upregulated bcl-xL in macrophages (Fig. 2) and a single stimulation could induce enough amounts of Bcl-xL to protect RAW264 from NO-induced apoptosis (Fig. 5), Bcl-xL may play a physiologic role in inducible self-defense mechanisms in macrophages against NO toxicity.

Induction mechanisms of bcl-xL initiated by LPS stimulation seem to be very similar to those of iNOS in activated macrophages since kinases (Fig. 1) and sensitivity to LPS stimulation (Fig. 2) for those inductions were very similar. Therefore, the signal-transduction pathways initiated by LPS stimulation may be shared between inductions of bcl-xL and iNOS. However, the costimulation of RAW264 with IFN-γ and LPS made different inducibilities between them (Fig. 2). Many DNA elements homologous to consensus sequences for the binding of transcription factors such as nuclear factor-κB and AP-1 are located in promoter regions of the iNOS gene (49), but not in those of the bcl-xL gene (50). Those elements in the iNOS promoter may be responsible for the synergistic augmentation. This is supported by the result that the induction of iNOS expression required synthesis of proteins such as c-fos and AP-1, which activated the bcl-xL promoter regions in activated macrophages (Fig. 3). Further study is required to elucidate the induction mechanisms of bcl-xL in activated macrophages.

In summary, bcl-xL but not bcl-2, was strongly induced in murine peritoneal macrophages stimulated with IFN-γ and LPS, and the induction was coincident with that in iNOS. Furthermore, overexpression of bcl-xL in RAW264 macrophage cell line inhibited or delayed apoptosis induced by the endogenous or the exogenous NO. These findings suggest that Bcl-xL is a physiologic molecule to contribute to the self-defense mechanisms against NO toxicity.

Acknowledgments

We thank Dr. S. Ohta (Nippon Medical School, Kawasaki, Japan) for Bcl-xL cDNA, and E. Furusawa and N. Fujita for secretarial assistance.

References


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