The Role of Antiapoptotic Bcl-2 Family Members in Endothelial Apoptosis Elucidated with Antisense Oligonucleotides*

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In this study, we utilized potent antisense oligonucleotides to examine the role of two Bcl-2 family members found in human umbilical vein endothelial cells (HUVEC). The first, A1, is thought to be a TNF-α-inducible cytoprotective gene, and the second, Bcl-XL, is constitutively expressed.

Inhibition of the constitutive levels of Bcl-XL caused 10–25% of the cell population to undergo apoptosis and increased the susceptibility of cells to treatment with low concentrations of staurosporin or ceramide. The caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp(OMe)-CH$_2$ prevented DNA fragmentation and ΔYm loss caused by Bcl-XL inhibition or Bcl-XL inhibition combined with staurosporin. However, disruption of ΔYm caused by Bcl-XL inhibition combined with ceramide treatment was not inhibited by benzyloxycarbonyl-Val-Ala-Asp(OMe)-CH$_2$, although DNA fragmentation was completely prevented. Taken together, these results demonstrate a direct protective role for Bcl-XL under normal resting conditions and under low level apoptotic challenges to HUVEC. Furthermore, Bcl-XL protects cells from caspase-dependent and -independent mechanisms of ΔYm disruption.

In contrast to Bcl-XL, A1 inhibition did not show a marked effect on the susceptibility of HUVEC to undergo apoptosis in response to TNF-α, ceramide, or staurosporin. These results demonstrate that although A1 may be a cytoprotective gene induced by TNF-α, it is not primarily responsible for HUVEC resistance to this cytokine.

The signaling pathways involved in apoptosis have been intensely studied in the last few years due to their role in many disease states including cancer and neurodegenerative disorders. One central player is the Bcl-2 family of proteins, which can either promote cell survival (Bcl-2, Bcl-XL, A1, Mcl-1, and Bcl-W) or promote cell death (Bax, Bak, Bel-XS, Bad, Bik, Bim, Hrk, Bok) (reviewed in Ref. 1). The relative amounts or equilibrium between these pro- and antiapoptotic proteins influence the susceptibility of cells to apoptosis. As such, many cancer cells up-regulate the antiapoptotic members as a mechanism to guard against programmed cell death. Although much work has been carried out studying the protective role of Bcl-2 and Bcl-XL in response to many different apoptotic stimuli, relatively little is known about the other antiapoptotic family members or the specific role of each isoform.

Endothelial cells play a pivotal role in modulating the inflammatory response. Stimulation of endothelial cells by tumor necrosis factor α (TNF-α) activates the transcription factor NF-κB, leading to induction of proinflammatory genes. TNF-α is also known to trigger programmed cell death, which in most cells is controlled by the simultaneous up-regulation of cytoprotective genes (Refs. 2–4; reviewed in Ref. 5). Because this anti-death activity requires de novo protein synthesis, cells resistant to the cytotoxic effects of TNF-α, including endothelial cells, can be rendered sensitive by pretreatment with either RNA or protein synthesis inhibitors. To date, several TNF-α-inducible cytoprotective genes have been identified and have been shown through overexpression techniques to protect cells, at least in part, from death induced by TNF-α and cycloheximide/actinomycin D. This growing family represents a diverse range of proteins and includes manganese superoxide dismutase (6), plasminogen activator inhibitor type 2 (7), the zine-finger protein A20 (8), the cyclin-dependent kinase inhibitor p21 (9), xiap, a member of the IAP family (10), IEX-1L, a protein of unknown function (11), the Bcl-2 family member A1 (12), and combinations of c-IAP-1, c-IAP-2, Traf1, and Traf2 (13).

We initiated studies aimed at addressing the role of A1 as a TNF-α-inducible cytoprotective gene and comparing its function to Bcl-XL, which is expressed constitutively in human umbilical vein endothelial cells (HUVEC). In order to help address the importance of each isoform in normal tissue, highly selective and potent antisense inhibitors were utilized in this study. We show that inhibition of A1 alone does not render HUVEC susceptible to TNF-α–induced apoptosis. However, inhibition of Bcl-XL caused HUVEC to undergo apoptosis and sensitized cells to treatment with either ceramide or staurosporin. Furthermore, Bcl-XL protected cells from both caspase-dependent and -independent mechanisms of ΔYm disruption.

Materials and Methods

Cells and Reagents—HUVEC were obtained from Clonetics (San Diego, CA) and cultivated in endothelial growth medium supplemented with 10% fetal bovine serum. Cells were used between passages 2 and 5. Opti-MEM, Lipofectin reagent, and fetal bovine serum were purchased from Life Technologies, Inc. TNF-α was obtained from R & D Research (Minneapolis, MN). Bcl-X antibody and the ribonuclease protection assay were from Pharmingen (San Diego, CA). Caspase 3 antibody was from Transduction Laboratories (Lexington, KY), and MitoTracker Orange CMTMRos was purchased from Molecular Probes (Eugene, OR). SDS-polyacrylamide gels were obtained from NOVEX (San Diego, CA). Staurosporin, C6-ceramide, benzyloxycarbonyl-Val-Ala-Asp(OMe)-CH$_2$ (Z-VAD.fmk), and carbonyl cyanide m-chlorophenylhydrazone were purchased from Calbiochem. Hybrid N+ nylon membranes and the ECL Plus detection kit were from Amersham Pharmacia Biotech.

Oligonucleotide Synthesis and Sequences Used—2′-O-Methoxymethyl chimeric antisense oligonucleotides were utilized for all experiments.

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1 The abbreviations used are: TNF-α, tumor necrosis factor α; HUVEC, human umbilical vein endothelial cells; ΔYm, mitochondrial transmembrane potential; Z-VAD.fmk, benzyloxycarbonyl-Val-Ala-Asp-CH$_2$F; PBS, phosphate-buffered saline; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.
The A1 antisense oligonucleotide and its control contained phosphodiester linkages in the 2′-O-methoxymethyl regions and phosphorothioate linkages in the 2′-deoxyoxymethyl regions, which support RNAse H activity in cells (14). The Bcl-XL antisense oligonucleotide and its control contained phosphorothioate linkages throughout. All oligonucleotides were synthesized using an Applied Biosystems 394B automated DNA synthesizer and purified as described previously (15, 16).

**Oligonucleotide and TNF-α Treatment of HUVEC—**HUVEC were treated with oligonucleotides at ~80% confluence (17, 18). Briefly, cells were washed three times with prewarmed (37 °C) Opti-MEM. Oligonucleotides were premixed with 10 μg/ml Lipofectin reagent in Opti-MEM at the desired concentration and applied to washed cells. HUVEC were incubated with the oligonucleotides for 4 h at 37 °C, after which the medium was removed and replaced with standard growth medium. For experiments carried out with A1 antisense oligonucleotide, the cells were allowed to recover 16–18 h after oligonucleotide treatment, prior to induction with TNF-α (10 ng/ml), C6-ceramide (5 μM), staurosporin (1 μM), or Z-VAD.fmk (100 μM). Because Bcl-XL is expressed constitutively, treatment with TNF-α (10 ng/ml), C6-ceramide (5–10 μM), staurosporin (1–2 μM), or Z-VAD.fmk (100 μM) was delayed 24 h after oligonucleotide treatment. This delay period allowed time for reduction in Bcl-XL protein prior to the addition of apoptotic stimuli.

**Northern Analysis—**Total cellular RNA was isolated either 4 h after TNF-α induction (A1 antisense experiments), or 24–48 h after oligonucleotide treatment (Bcl-XL antisense experiments) utilizing Quigen RNeasy Kit. Isolated RNA was separated on a 1% agarose/formaldehyde gel, transferred to a Hybond N nylon membrane over-night, and hybridized with random primed 32P-labeled human G3PDH cDNA to confirm equivalent loading of RNA samples. Membranes were treated for a minimum of 2 h with specific primary antibody followed by incubation with secondary antibody conjugated to horseradish peroxidase. Visual-}

**Hydroploidy Apoptosis Assay—**Cells were treated with oligonucleotides and apoptosis inducers as described. Both floating and adherent cells were harvested 24–48 h after treatment; washed once with 1 ml of PBS, 5 mM EDTA; and fixed with 1 ml of 70% EtOH while vortexing. Cells were pelleted by centrifugation, washed once with 1.0 ml of PBS, 5 mM EDTA, and resuspended with 0.3–1.0 ml Propidium Iodide mix (250 μg/ml propidium iodide, 5 μg/ml RNAse A, 1 × PBS, and 5 mM EDTA). After incubation in the dark for 1 h at room temperature, the cells were analyzed on a Becton Dickinson FACscan, and apoptotic cells (sub-G₁ population) were quantified.

**Mitochondrial Transmembrane Potential (ΔYm)—**To evaluate the ΔYm, cells were incubated with the cationic lipophilic dye MitoTracker orange CMTMROs (150 nM) for 15 min at 37 °C in the dark (20). Control cells were simultaneously treated with 50 μM of the protophorphyrin, carbonyl cyanide m-chlorophenylhydrazone, which disrupts ΔYm. Both adherent and floating cells were collected, washed once with PBS, 2% bovine serum albumin, and fixed in 1 ml of PBS containing 4% paraformaldehyde for 15 min while shaking at room temperature. Fixed cells were stored in the dark at 4 °C for 1 day prior to analysis by flow cytometry. The percentage of cells with decreased fluorescence, and therefore with reduced ΔYm (compared with control cells not treated with oligonucleotides or apoptotic inducers), were determined and indicated on the bar graphs as percentage of cells gated. Cells treated with carbonyl cyanide m-chlorophenylhydrazone generally gave greater than 70% cells gated and showed a shift of 0.5 log units to a lower fluorescence.

**Caspase 3 Activity—**Caspase 3 activity was measured using Caspase 3 colorimetric assay kit from CLONTECH (Palo Alto, CA). Briefly, whole cell lysates were obtained, protein concentrations were determined, and equal amounts of protein were added to 50 μM substrate (Asp-Glu-Val-Asp-p-nitroanilide) in assay buffer. Samples were incubated at room temperature and analyzed using a spectrophotometer (OD 418).

**RESULTS**

**Bcl-2 Family Members in HUVEC—**Ribonuclease protection assay was utilized to identify Bcl-2 family members present in basal and TNF-α-treated HUVEC. High levels of the antiapoptotic members Bcl-XL and Mcl-1 were observed in both basal and induced cells, while Bcl-2 was not detected (Fig. 1). In contrast, the antiapoptotic member A1 was found in low abundance in basal HUVEC but increased ~10-fold following TNF-α treatment. Maximal induction of A1 was observed ~4 h after treatment. Interestingly, Bcl-XL was down-regulated approximately 20% upon treatment with TNF-α.

### Table 1

**Sequence and chemistry of oligonucleotides**

All oligonucleotides utilized were chimeras with 2′-deoxy or 2′-O-methoxymethyl modifications. Sequences containing the 2′-O-methoxymethyl modifications are shown in parentheses. The A1 antisense oligonucleotide and its control contained phosphodiester linkages in the 2′-O-methoxymethyl regions and phosphorothioate linkages in the 2′-deoxyoxymethyl region (as indicated by O or S). The Bcl-XL antisense oligonucleotide and its control contained phosphorothioate linkages throughout.

| Target | Sequence | Position on mRNA |
|--------|----------|-----------------|
| A1     | (A,T,T,C,G,T,T) T,T,C,C,A,T,A,T,A,T,C,A, (G,T,C,A,G) | 648** |
| Control| (A,T,T,C,G,T,T) T,C,C,T,A,T,T,A,A, (G,T,C,A,G) | 648** |
| Bcl-XL | (C,T,A,G,G) C,T,T,T,C,A,C,G,C, (G,C,A,T,T) | 580** |
| Control| (G,A,G,A,C) C,G,T,A,C,C,T,C,C, (G,C,A,T,T) | 580** |

*Accession numbers for A1 and Bcl-XL are U29680 and Z33115, respectively.*

![Fig. 1](image-url)
Inhibition of A1 mRNA through Antisense Oligonucleotide Treatment—An effective antisense oligonucleotide directed toward A1 was identified from a screen targeting 14 distinct positions on the A1 mRNA, extending from the 5'-UTR to the 3'-UTR using methods as described previously (21). All oligonucleotides were 20 nucleotides in length containing chimeric 2'-O-methoxyethyl modifications on a mixed backbone of phosphodiester and phosphorothioate linkages (Table I). The most effective A1 antisense oligonucleotide was directed toward the 3'-UTR, immediately downstream from the stop codon, and was utilized in all subsequent experiments.

Fig. 2 shows the concentration-dependent decrease of the A1 mRNA with increasing amounts of A1 antisense oligonucleotide, while a mismatch control oligonucleotide failed to show a reduction, demonstrating a sequence specific inhibition. The apparent IC_{50} for the A1 antisense compound was less than 10 nM. Treatment of HUVEC with 50 nM of the A1 antisense oligonucleotide reduced the A1 mRNA to basal levels, completely inhibiting the TNF-α-induced component. Reductions in A1 mRNA to basal levels were observed up to 30 h after antisense treatment (data not shown).

Inhibition of Bcl-XL mRNA and Protein through Antisense Oligonucleotide Treatment—A potent, sequence-specific antisense oligonucleotide directed toward Bcl-XL has previously been identified and shown to decrease Bcl-XL mRNA and protein levels in both human keratinocytes and A549 cells. Here we tested its ability to inhibit Bcl-XL mRNA in HUVEC. As shown in Fig. 3, treatment of HUVEC with 100 nM of the Bcl-XL antisense oligonucleotide decreased the A1 mRNA to basal levels, completely inhibiting the TNF-α-induced component. Reductions in A1 mRNA to basal levels were observed up to 30 h after antisense treatment (data not shown).

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![Fig. 2. Dose-dependent inhibition of A1 mRNA by antisense oligonucleotide. HUVEC were incubated with increasing concentrations of a specific A1 antisense oligonucleotide. After a recovery period of 16 h, cells were induced with TNF-α for 4 h, and total RNA was extracted. Northern analysis was carried out, and the membrane was probed sequentially with a 32P-labeled A1 probe and a G3PDH 32P-labeled probe (shown in the bottom panel). The amount of specific RNA in each lane was quantified. The graph shows the average of three separate experiments.](image1)

![Fig. 3. Dose-dependent inhibition of Bcl-XL mRNA by antisense oligonucleotide. HUVEC were incubated with the increasing concentrations of a specific Bcl-XL antisense or mismatch oligonucleotide. Cells were harvested 24 h later, and total RNA was extracted. Northern analysis was carried out, and the membrane was probed sequentially with a Bcl-XL 32P-labeled probe and a G3PDH 32P-labeled probe (shown in the bottom panel). The amount of specific RNA in each lane was quantified, and the figure shows results from a typical experiment.](image2)

![Fig. 4. Inhibition of Bcl-XL protein expression. Cells were treated with Bcl-XL antisense or mismatch oligonucleotide (50 nM) and harvested at 16, 24, or 48 h. Bcl-XL protein levels were determined by Western blot. The Bcl-XL band migrates at ~30 kDa.](image3)

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2 Taylor, J. K., Zhang, Q. Q., Monia, B. P., Marcusson, E., and Dean, N. M. (1999) *Oncogen*, in press.
could represent changes in the phosphorylation state of Bcl-XL. If the Bcl-XS protein were present, it should migrate well below the Bcl-XL band under our conditions. Thus, the Bcl-XL antisense compound was able to decrease Bcl-XL protein levels to roughly 5% of amounts in untreated cells.

Specificity of Antisense Oligonucleotide Treatment—In order to demonstrate the specificity of A1 and Bcl-XL antisense oligonucleotides, HUVEC were treated with A1 antisense oligonucleotide, A1 control, Bcl-XL antisense oligonucleotide, or Bcl-XL control, in the presence or absence of TNF-α. Northern analysis was carried out, and the membrane was probed sequentially with A1-, Bcl-XL-, and G3PDH-specific probes. The A1 antisense compound was effective in reducing A1 mRNA without affecting the Bcl-XL mRNA. Likewise, the Bcl-XL antisense compound specifically reduced Bcl-XL mRNA without affecting A1 mRNA (Fig. 5). In addition, there was no effect on either target with the control oligonucleotides. These results demonstrate that the antisense oligonucleotides utilized here can specifically inhibit the target mRNA without affecting other Bcl-2 family members.

HUVEC Viability after Inhibition of Endogenous A1 and Bcl-XL Protein: Basal Versus TNF-α Induction—Because A1 is induced by TNF-α treatment, its role in protecting HUVEC from TNF-α-induced apoptosis has been suggested (12). To address this point, cells were treated with 50 nM A1 antisense oligonucleotide or control oligonucleotide and subsequently induced with TNF-α. Apoptotic cells with fragmented DNA were identified by flow cytometry analysis. As shown in Fig. 6A, inhibition of the TNF-α-induced A1 mRNA did not significantly affect the number of apoptotic cells observed after TNF-α treatment. However, cells treated with a combination of TNF-α and cycloheximide, which prevents the up-regulation of all TNF-α-inducible cytoprotective genes, resulted in over 50% cell death under these conditions. These results suggest that A1 is not solely responsible for protection of HUVEC from TNF-α-induced programmed cell death.

Similar experiments were also carried out with the Bcl-XL antisense compound. Inhibition of Bcl-XL protein in basal HUVEC caused 10–25% of the cell population to undergo apoptosis (Fig. 6B). Thus, Bcl-XL may play an important role in the basal viability of endothelial cells. On the other hand, inhibition of the Bcl-XL protein did not render cells more sensitive to TNF-α treatment (i.e. the difference between Bcl-XL and control remained the same in the presence of TNF-α), indicating that

Bcl-XL is not critical in protecting cells from this apoptotic stimuli.

Because inhibition of either A1 or Bcl-XL protein did not render HUVEC more susceptible to TNF-α induced apoptosis, we investigated whether simultaneous inhibition of both proteins would result in increased apoptosis. Therefore, HUVEC were treated with both A1 and Bcl-XL antisense compounds, allowed to recover for 24 h, and then treated with or without TNF-α. Fig. 6C shows that simultaneous inhibition of A1 and Bcl-XL did not significantly render HUVEC more susceptible to TNF-α treatment.
with 1 nM staurosporin or 5 μM C6-ceramide alone did not significantly affect the cellular DNA fragmentation. However, cells with decreased levels of Bcl-XL protein showed increased sensitivity toward these low concentrations of apoptotic stimuli (Figs. 7 and 8A). In contrast, cells pretreated with the A1 antisense compound showed only a slight increase in DNA fragmentation after treatment with TNF-α in the presence of either staurosporin or ceramide (Fig. 9A). As shown in Fig. 9A, HUVEC were more sensitive to ceramide treatment when carried out in the presence of TNF-α as described previously (23).

One of the major mechanisms of Bcl-2-mediated cytoprotection is by protecting mitochondrial function (reviewed in Refs. 24 and 25). Therefore, we investigated the effect of A1 and Bcl-XL inhibition on the ΔYm. A reduction in ΔYm has been shown to accompany apoptosis in many experimental models and can be monitored by the potential-sensitive, fluorescent dye CMTMRos. To confirm that CMTMRos was sensitive to mitochondrial transmembrane depolarization, control cells were treated with 50 μM of the mitochondrial uncoupling agent carbonyl cyanide m-chlorophenylhydrazone. As shown in Fig. 8C, treatment of HUVEC with Bcl-XL antisense compound resulted in a reduction in the ΔYm. This reduction was potentiated by the combination of Bcl-XL inhibition and treatment with either staurosporin or ceramide. Thus, endogenous Bcl-XL may function by maintaining the mitochondrial integrity of resting HUVEC, where loss of Bcl-XL results in greater susceptibility to mitochondrial dysfunction. In contrast to these results, inhibition of A1 did not effect the ΔYm, after treatment with either TNF-α alone or TNF-α in combination with ceramide or staurosporin (Fig. 9C).

Involvement of Caspases in HUVEC Apoptosis Resulting from Bcl-XL Inhibition—HUVEC were treated with the A1 or Bcl-XL antisense oligonucleotides or controls, in the presence or absence of Z-VAD.fmk, a broad-based cell-permeable caspase inhibitor. As shown in Fig. 8B, Z-VAD.fmk completely inhibited the DNA fragmentation resulting from Bcl-XL inhibition alone or from Bcl-XL inhibition in the presence of ceramide or staurosporin. Similar results were also obtained using Z-DEVD.fmk, a known inhibitor of caspases 3, 7, and 8 (data not shown). Besides affecting DNA fragmentation, Z-VAD.fmk also inhibited ΔYm loss induced by Bcl-XL antisense oligonucleotide or Bcl-XL antisense oligonucleotide and staurosporin (Fig. 8D). However, inhibition of ΔYm was not observed when cells deficient in Bcl-XL protein were treated with ceramide. Bcl-XL...
conserved, a number of recent reports have identified several candidates. One such candidate, A1, was originally identified in endothelial cells expressing high levels of A1, through retrovirus-mediated expression or molecular mechanism of each isoform, since many cells express more than one. Through ribonuclease protection assay analysis, we show that endothelial cells express Bcl-XL and Mcl-1 constitutively, while A1 is found in very low amounts in basal cells but can be up-regulated by TNF-α. Therefore, we initiated studies aimed at addressing and comparing the normal physiological role between two of these family members, A1 and Bcl-XL, by utilizing specific antisense oligonucleotides to inhibit the endogenous levels of each protein.

Although the identity of the survival factor(s) responsible for halting the TNF-α death pathway remains to be firmly established, a number of recent reports have identified several candidates. One such candidate, A1, was originally identified in phorbol ester-stimulated endothelial cells and shown to be induced by the inflammatory cytokines TNF-α and interleukin-1 (27). Further experiments demonstrated that endothelial cells expressing high levels of A1, through retrovirus-mediated transfer, were protected against cell death initiated by TNF-α plus actinomycin D (12). This protection was also observed with overexpression of Bcl-XL (12). However, others have reported that overexpression of either A1 or Bcl-XL did not rescue HUVEC from death induced by TNF-α plus cycloheximide, although both effectively inhibited apoptosis induced by TNF-α plus ceramide. Thus, we demonstrate that treatment of HUVEC with a highly specific and potent antisense oligonucleotide directed toward A1 completely inhibited the TNF-α-inducible component of the A1 mRNA. However, this inhibition did not render cells significantly more susceptible to apoptosis by TNF-α. Furthermore, cells with decreased levels of Bcl-XL, or both Bcl-XL and A1, continued to show resistance to TNF-α treatment. Thus, A1 and Bcl-XL do not appear to play critical roles in protecting endothelial cells from TNF-α.

In contrast, inhibition of the endogenous levels of Bcl-XL resulted in a substantial amount of cell death in basal cells. In addition, Bcl-XL-deficient cells were rendered sensitive to concentrations of ceramide and staurosporin that were without effect in cells expressing normal levels of Bcl-XL. Thus, Bcl-XL may function physiologically as a protective gene that, under normal resting conditions, helps endothelial cells maintain their quiescent phenotype and perform their normal barrier functions even in the presence of low levels of stimulants (26). This would be critical for endothelial cells in vivo, which are constantly assaulted by noxious compounds such as oxidized low density lipoproteins or cytokines. Our results are consistent with overexpression data indicating that Bcl-XL protects cells from assault from a wide range of apoptotic stimulants, including ceramide (12, 28) and staurosporin (29). It is interesting to note that as the concentrations of staurosporin and ceramide increased, the differences observed between normal and Bcl-XL-inhibited HUVEC slowly disappeared (data not shown). These results suggest that endogenous Bcl-XL can protect HUVEC from apoptotic stimuli up to a threshold level

Antisense Oligonucleotide Inhibition of Bcl-XL and A1

DISCUSSION

To date, much work has been carried out studying the protective role of Bel-2 and Bcl-XL, while relatively little is known about the other antiapoptotic Bcl-2 family members A1, Bcl-W, and Mcl-1. One unresolved question has been the specific function or molecular mechanism of each isoform, since many cells express more than one. Through ribonuclease protection assay analysis, we show that endothelial cells express Bcl-XL and Mcl-1 constitutively, while A1 is found in very low amounts in basal cells but can be up-regulated by TNF-α. Therefore, we initiated studies aimed at addressing and comparing the normal physiological role between two of these family members, A1 and Bcl-XL, by utilizing specific antisense oligonucleotides to inhibit the endogenous levels of each protein.

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![Fig. 8](https://example.com/f8.png) **Effect of Bcl-XL inhibition, ceramide, and staurosporin on DNA fragmentation and ΔYm in the presence and absence of Z-VAD.fmk.** Cells were treated with Lipofectin reagent alone (a), 50 nM Bcl-XL antisense (b) or 50 nM control (c) as indicated. After 24 h, cells were treated with ceramide (10 μM), staurosporin (2 μM), or Z-VAD.fmk (100 μM) as indicated, followed by an additional 24-h incubation. Harvested cells were analyzed by fluorescence-activated cell sorting for hypodiploidy (A and B) or ΔYm (C and D) as described under "Materials and Methods." Results shown are representative of five independent experiments.
were induced with TNF-

a

nM A1 antisense (24 and 25). More specifically, these proteins are thought to

function through maintaining the mitochondrial permeability transition pore opening (and therefore protecting mitochondrial function (reviewed in Refs. 24 and 25). This may make sense physiologically, since a high level of ceramide or staurosporin. However, Z-VAD.fmk loss and apoptosis in a caspase-independent fashion (37).

Taken together, our data are consistent with endogenous Bcl-XL functioning by maintaining mitochondrial integrity of resting HUVEC, where loss of Bcl-XL results in greater susceptibility to mitochondrial dysfunction. The protection of 

Apaf-1, resulting in the activation of caspase 9 and subsequently caspase 3. It is now apparent that in some systems, cytochrome c release occurs prior to loss in 

Ym and that the caspase 3 activity was determined using 50 nM DEVD-p-nitroanilide substrate and recording OD 418.

These results can be explained in view of the current models. When cytochrome c is released into the cytosol, it can bind to caspase-independent mechanisms. These results demonstrate a definitive role for Bcl-XL in these processes and are complementary to overexpression data obtained previously.
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