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## p38 MAPK mediates TNF-induced apoptosis in endothelial cells via phosphorylation and downregulation of Bcl-x<sub>L</sub>

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### Abstract

The role of p38 mitogen-activated protein kinase (MAPK) in apoptosis is a matter of debate. Here, we investigated the involvement of p38 MAPK in endothelial apoptosis induced by tumor necrosis factor  $\alpha$  (TNF). We found that activation of p38 MAPK preceded activation of caspase-3, and the early phase of p38 MAPK stimulation did not depend on caspase activity, as shown by pretreatment with the caspase inhibitors z-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk) and Boc-Asp(OMe)-fluoromethylketone (BAF). The p38 MAPK inhibitor SB203580 significantly attenuated TNF-induced apoptosis in endothelial cells, suggesting that p38 MAPK is essential for apoptotic signaling. Furthermore, we observed a time-dependent increase in active p38 MAPK in the mitochondrial subfraction of cells exposed to TNF. Notably, the level of Bcl-x<sub>L</sub> protein was reduced in cells undergoing TNF-induced apoptosis, and this reduction was prevented by treatment with SB203580. Immunoprecipitation experiments revealed p38 MAPK-dependent serine–threonine phosphorylation of Bcl-x<sub>L</sub> in TNF-treated cells. Exposure to lactacystin prevented both the downregulation of Bcl-x<sub>L</sub> and activation of caspase-3. Taken together, our results suggest that TNF-induced p38 MAPK-mediated phosphorylation of Bcl-x<sub>L</sub> in endothelial cells leads to degradation of Bcl-x<sub>L</sub> in proteasomes and subsequent induction of apoptosis.

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**Keywords:** Apoptosis; Bcl-x<sub>L</sub>; Endothelial cells; Mitochondria; p38 MAP kinase; Phosphorylation; TNF

### Introduction

The pleiotropic cytokine known as tumor necrosis factor- $\alpha$  (TNF) is produced by many types of cells, and it was originally identified from its cytotoxic effects. In addition to its ability to induce cell death, TNF can elicit a wide range of physiological responses, including inflammatory reac-

tions and cell proliferation and differentiation [1]. TNF exerts its pleiotropic and cell-dependent effects by activating multiple cellular signal transduction pathways [2]. Ligand and trimerization of TNF-R1 lead to recruitment of several adapter signaling proteins, among them Fas-associated death domain protein (FADD). FADD recruits procaspase-8 molecules and is responsible for activation of the self-amplifying caspase cascade, an effect that is necessary for the process of apoptosis [3,4]. Stimulation of TRAF2, on the other hand, is assumed to initiate activation of three other proteins: p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), and the transcription factor Nuclear Factor  $\kappa$ B (NF- $\kappa$ B) [2].

The caspases are cysteine proteases that always cleave their substrates at an aspartic residue. The important upstream/initiator caspase-8 is directly linked to death receptors such as the TNF and Fas (CD95) receptor complexes. Caspase-3, which is an effector caspase that acts downstream of caspase-8, has been proposed to be the most important enzyme in the process of apoptosis [5]. A large

**Abbreviations:** BAF, Boc-Asp(OMe)-fluoromethylketone; cFLIP, cellular FLICE-inhibitory protein; CHX, cycloheximide; DISC, death-inducing signaling complex; DQMD-fmk, z-Asp-Gln-Met-Asp(OMe)-fluoromethylketone; ERK, extracellular signal regulated kinases; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; mAb, monoclonal antibody; pAb, polyclonal antibody; PARP, poly-(ADP-ribose) polymerase; PBS, phosphate-buffered saline; TBST, Tris-buffered saline containing Tween 20; TNF, tumor necrosis factor  $\alpha$ ; VAD-fmk, z-Val-Ala-Asp(OMe)-fluoromethylketone.

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number of caspase substrates have been identified in apoptotic cells, and most of them play fundamental roles in the cytoskeleton, in signaling pathways, or in DNA repair [6].

The MAPK pathway is one of the major systems used by eukaryotic cells to transduce extracellular signals into intracellular responses. A common feature of all MAPK isoforms is that they are phosphorylated on both threonine and tyrosine residues by a dual-specificity serine–threonine MAPK kinase (MKK), which is in turn phosphorylated and activated by an upstream MKK kinase (MKKK). Once activated, MAPKs can phosphorylate and activate other kinases or nuclear proteins, such as transcription factors, in either the cytoplasm or the nucleus. The p38 MAPK family includes four members that are designated p38 $\alpha$ , - $\beta$ , - $\gamma$ , and - $\delta$  MAPK; only the  $\alpha$ ,  $\beta$ , and  $\delta$  proteins are produced by endothelial cells [7]. Some reports in the literature disagree about the role of MAPKs in apoptosis. Early studies of fibroblasts suggested that extracellular signal-regulated kinase (ERK; p42/p44 MAPK) activity promotes cell survival and inhibits apoptosis, but they also indicated that p38 MAPK promotes apoptosis [8,9]. Experiments on endothelial cells have demonstrated that vascular endothelial growth factor (VEGF) exerts its anti-apoptotic effect via activation of ERK [10] and upregulation of the anti-apoptotic protein Bcl-2. Furthermore, it has been shown that Fas-induced activation of ERK prevents Fas-mediated apoptosis [11]. However, concerning the role of p38 MAPK, we have observed [12], as have others [13–16], that this kinase may also protect some types of cells from apoptosis. A well-known downstream target of p38 MAPK is NF- $\kappa$ B [17,18], which has been found to induce transcription of several anti-apoptotic genes, including the one encoding FLICE-inhibitory protein (cFLIP; Ref. [19]). Indeed, there is accumulating evidence that the involvement of p38 MAPK in apoptosis is both cell-type and stimulus dependent. Considering endothelial cells, some researchers have shown that induction of apoptosis by treatment with thrombospondin-1 [20] or high levels of D-glucose [21] requires p38 MAPK activity, whereas others have reported that inhibition of apoptosis by estradiol is mediated via activation of p38 MAPK [22].

Proteins of the Bcl-2 family play important roles in most types of apoptosis, and their primary target of action is the mitochondrion. Bax and Bak are two members of this family that are responsible for modulating the permeability of mitochondrial membranes, an effect that results in the release of cytochrome *c* and subsequent activation of caspase-9, and ultimately also activation of effector caspases [23]. The pro-apoptotic proteins Bax and Bak are regulated by Bid, which functions as an allosteric activator [24]. Bcl-2 and its close homolog Bcl-x<sub>L</sub> potentially inhibit apoptosis in response to many cytotoxic insults, and it is assumed that this blocking effect occurs via formation of heterodimers with pro-apoptotic members of the Bcl-2 family. Thus, the ratio of pro-apoptotic to anti-apoptotic Bcl-2 family proteins is a critical factor that determines cellular susceptibility to

apoptosis. Two other anti-apoptotic Bcl-2 proteins, A1 and Mcl-1, have also been detected in endothelial cells [25]. Vascular endothelium is a major target of the actions of TNF, including the induction of adhesion and activation of leukocytes, coagulation, and apoptosis [26,27]. Many of the TNF-induced pathways described thus far have proven to be cell-type specific; therefore, it is important that observations made in other kinds of cells be confirmed or ruled out in endothelial cells. The objective of the present study was to elucidate the role of p38 MAPK in TNF-induced endothelial apoptosis. Our results suggest that p38 MAPK mediates TNF-induced apoptosis of endothelial cells via phosphorylation and downregulation of Bcl-x<sub>L</sub>.

## Materials and methods

### Materials

Recombinant human TNF- $\alpha$  and anti-Bid Ab were purchased from R&D Systems (Abingdon, UK), and Asp-Glu-Val-Asp-aminomethylcoumarin (DEVD-amc) was from Upstate Biotechnology (Lake Placid, NY). SB203580 (inhibitor of  $\alpha$  and  $\beta$  isoforms of p38 MAPK), Nonidet P-40, lactacystin, anti-p38 MAPK polyclonal antibody (pAb) (recognizing p38 $\alpha$  MAPK), and anti-porin monoclonal antibody (mAb) were obtained from Calbiochem (La Jolla, CA). The caspase inhibitors z-Val-Ala-Asp(Ome)-fluoromethylketone (zVAD-fmk), z-Asp-Gln-Met-Asp(Ome)-fluoromethylketone (zDQMD-fmk), and Boc-Asp(Ome)-fluoromethylketone (BAF) were supplied by Enzyme Systems Products (Livermore, CA). Anti-A1 pAb, anti-Bcl-x<sub>L</sub> pAb, and an enhanced chemiluminescence (ECL) kit were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). NaVO<sub>4</sub> was from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Leupeptin, Pefabloc SC, and pepstatin were from Roche Diagnostics (Mannheim, Germany). Coomassie Plus Protein Assay reagent was from Pierce (Rockford, IL). Anti-caspase-8 mAb, anti-caspase-3 mAb, and anti-cFLIP mAb were purchased from Alexis Biochemicals (Läufelfingen, Switzerland). Anti-Bcl-2 mAb, anti-phospho-serine–threonine mAb, anti-Mcl-1 mAb, and anti-Bak mAb were from BD Biosciences (Heidelberg, Germany), and anti-poly-(ADP-ribose) polymerase (PARP) mAb was bought from BIOMOL Research Laboratories (Plymouth Meeting, PA). Anti-phospho-p38 MAPK Ab (detects all isoforms of p38 MAPK that have been activated by dual phosphorylation at Thr180 and Tyr182), anti-phospho-JNK Ab, and anti-JNK Ab were obtained from Cell Signaling Technology (Beverly, MA), and anti-Bax mAb was from Biosource (Nivelles, Belgium). HRP-coupled goat anti-rabbit and goat anti-mouse immunoglobulins were from Dako A/S (Glostrup, Denmark), and trypan blue was purchased from Gibco (Invitrogen, Paisley, UK). SDS polyacrylamide gel electrophoresis reagents and equipment were from Bio-Rad (Rockford, IL). Densitometric analysis of

Western blots and autoradiographs was done with a Fluor-S Multimager from Bio-Rad. Reagents not listed here were obtained from Sigma (St. Louis, MO), unless otherwise stated.

#### Cell culture

The endothelium-derived permanent human cell line EA.hy926 [28] was maintained in Dulbecco's Modified Eagle's Medium supplemented with 2 mM L-glutamine, 50 IU/ml penicillin–streptomycin (all from Gibco, Invitrogen), and 10% heat-inactivated fetal bovine serum (PAA Laboratories, Linz, Austria). Cells were maintained at 37°C in a 5% CO<sub>2</sub> humidified incubator and were subcultured every 5 days using trypsin-EDTA solution for endothelial cells. In the inhibitor experiments, cells were preincubated with inhibitors for 30 min and thereafter exposed to TNF; the inhibitors were also present during exposure to TNF.

#### Caspase-3 assay

Caspase-3 activity was analyzed with a fluorescence spectrophotometric assay using the fluorogenic peptide DEVD-amc as a substrate. After appropriate incubations, cells were harvested by scraping into 300 µl of caspase lysis buffer (10 mM Tris–HCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> [pH 7.5], 130 mM NaCl, 0.1% Triton X-100, and 10 mM NaPP<sub>3</sub>) on ice. Floating cells were collected, pelleted, and pooled with the lysate. The assay was performed in white, 96-well Fluoronunc™ Polysorp plates (Nunc A/S, Roskilde, Denmark). Lysates (50-µl aliquots) were added to the reaction wells together with 200 µl of HEPES buffer (20 mM HEPES [pH 7.5], 10% glycerol, and 2 mM dithiothreitol) and 4 µl of DEVD-amc (dissolved in DMSO). The reaction mixture was incubated at 37°C for 1 h, and thereafter analyzed for amc fluorescence with a Fluostar plate reader (BMG Lab Technologies, Germany) using excitation and emission wavelengths of 390 and 460 nm, respectively. The fluorescence data given in the figures are net values corrected by subtraction of background values. The protein content of lysates was determined using the Coomassie Plus assay.

#### Analysis of cell viability

After appropriate incubations in 60-mm culture dishes, cells were trypsinized. Floating cells were collected, pelleted, and pooled with trypsinized cells, and viability was assessed by trypan blue exclusion. Briefly, 10 µl of cell suspension was combined with 90 µl of 0.4% sterile filtered trypan blue, and cell counts were performed using a hemacytometer.

#### Subcellular fractionation

Isolation of mitochondrial and cytosolic/microsomal sub-fractions [29] was achieved as follows. Adherent cells were

washed in buffer D (100 mM sucrose, 1 mM EGTA, and 20 mM MOPS [pH 7.4]) and then scraped into buffer E (Buffer D supplemented with 5% Percoll, 0.01% digitonin, and a protease inhibitor cocktail comprising 10 µM aprotinin, 10 µM pepstatin A, 10 µM leupeptin, and 4 mM Pefabloc). The resulting lysates were transferred to Eppendorf tubes and incubated on ice for 15 min. Thereafter, cells and nuclei that were still intact were pelleted at 2500 × g for 10 min at 4°C. The supernatant was centrifuged at 15,000 × g for 15 min, and the pellet containing mitochondria was resuspended in buffer F (300 mM sucrose, 1 mM EGTA, 20 mM MOPS [pH 7.4], and the protease inhibitor cocktail specified above). The Coomassie Plus protein assay was used to determine the protein concentration of each subfraction.

#### Western blot analysis

After various incubations in 60-mm culture dishes, floating cells were collected and pelleted, and adherent cells were harvested by scraping into 200 µl of Western lysis buffer (150 mM NaCl, 20 mM Tris–HCl [pH 7.4], 5 mM EDTA, 2 mM NaVO<sub>4</sub>, 4 µg/ml leupeptin, 2 mM Pefabloc SC, and 1% NP-40). The pelleted cells were pooled with the lysate, and 50 µl of 5× sample buffer (312 mM Tris–HCl [pH 6.8], 10% SDS, 50% glycerol, 0.01% bromophenol blue, and 150 mM dithiothreitol) was added to each sample. The cells were subsequently heated to 90°C for 5 min in a heating block. Proteins were separated under reducing conditions in SDS-polyacrylamide gels and then Western blotted onto polyvinylidene difluoride (PVDF) filters at 100 V for 80 min. Next, the blots were blocked with Tris-buffered saline/Tween (TBST; 50 mM Tris base, 150 mM NaCl, and 0.05% Tween 20) containing 5% dry milk powder (or 3% bovine serum albumin, when anti-phospho-serine–threonine Ab was used) and thereafter incubated for 1–2 h with respective antibodies. After that procedure, the blots were washed and incubated with HRP-coupled secondary Ab, and bound Ab was detected by use of an ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

#### Immunoprecipitation

For immunoprecipitation of Bcl-x<sub>L</sub>, EA.hy926 cells were cultured in 100-mm culture dishes, treated as indicated in the figures, and then scraped into 800 µl of immunoprecipitation buffer (150 mM NaCl, 10 mM Tris–HCl [pH 7.4], 1 mM EDTA, 1 mM EGTA, 2 mM orthovanadate, 2 mM Pefabloc, and 1% NP-40). The resulting lysate was pre-cleared with Protein A Sepharose for 30 min, and Bcl-x<sub>L</sub> was immunoprecipitated overnight with an anti-Bcl-x<sub>L</sub> mAb (4 µg/ml). Thereafter, the antibody was pulled down by incubation with protein G agarose for 90 min. The immunoprecipitate was washed three times with the above-mentioned buffer, resuspended in 2× sample buffer (125 mM Tris–HCl [pH 6.8], 4% SDS, 20% glycerol, 0.05% bromophenol blue, and 100 mM dithiothreitol), heated to 90°C for

5 min, and then loaded onto SDS-polyacrylamide gels and subjected to Western blotting.

## Results

### *Caspase-3 activation in endothelial cells undergoing TNF-induced apoptosis*

Treatment of EA.hy926 cells with TNF in combination with cycloheximide (CHX) caused pronounced activation of caspase-3 as indicated by the DEVD-amc cleavage assay (Fig. 1A). The activity of caspase-3 began to increase after 2 h and reached a maximum level after about 8 h of exposure to TNF, and the proteolytic activation with formation of active p17/p12 exhibited similar kinetics (Fig. 1B). TNF alone did not induce caspase-3 activity, measured as

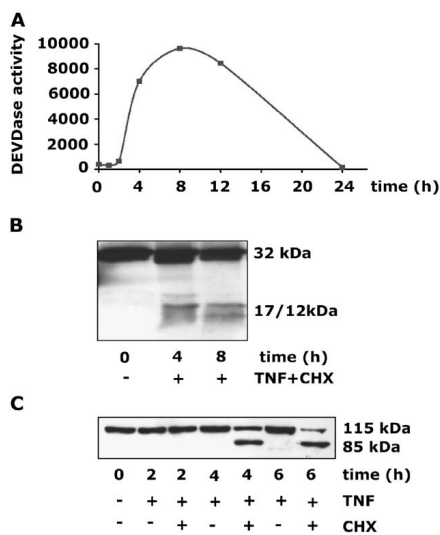


Fig. 1. Activation of caspase-3 in TNF-stimulated EA.hy926 cells. Cells were treated with both TNF (10 ng/ml) and cycloheximide (CHX, 1  $\mu$ g/ml), unless otherwise indicated. (A) Cells were treated with TNF and CHX for the indicated times, after which caspase-3 activity was measured as cleavage of DEVD-amc in a fluorescence plate reader assay, analyzing equal amounts of protein from each sample, as described in Materials and methods. Treatment with TNF alone did not induce any activation of DEVD-amc cleavage (data not shown). (B) Cells were treated with TNF and CHX for the times indicated and then processed for Western blotting. Proteolytic activation of pro-caspase-3 was detected using an anti-caspase-3 polyclonal Ab. The 32-kDa band represents intact pro-caspase-3, and the 17/12-kDa bands are active caspase-3 fragments. (C) Cells were exposed to TNF with or without CHX, as indicated, and then processed for Western blotting. Cleavage of PARP (116 kDa) and production of the 85-kDa fragment were detected with an anti-PARP mAb. The illustrated blots are representative of three independent experiments.

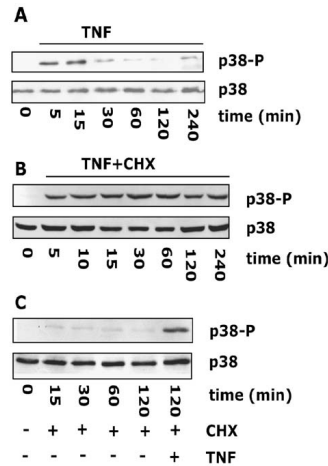


Fig. 2. Phosphorylation and activation of p38 MAPK in EA.hy926 cells undergoing TNF-induced apoptosis. Cells were exposed to TNF alone (A), TNF and CHX (B), or CHX alone (C) for the indicated times, and then processed for Western blotting. An anti-phospho-p38 MAPK Ab was used to detect the phosphorylated form of p38 MAPK (p38-P) on Western blots, after which the PVDF membrane was stripped and reprobed with an Ab against total cellular p38 MAPK as a loading control. Note that the last lane in (C) is a sample co-treated with TNF and CHX, which is shown as a positive control. The illustrated blots are representative of 3–4 independent experiments.

DEVD-amc cleavage (data not shown) or as poly(ADP-ribose) polymerase (PARP) cleavage (Fig. 1C). Cycloheximide alone did not have any effect on caspase-3 activity (data not shown). Exposure to TNF and cycloheximide for 4 h led to >50% cleavage of nuclear PARP, whereas the majority of this protein was proteolyzed after 6 h of treatment (Fig. 1C). These results agree with previous findings showing that, for most cultured cell lines, a cytotoxic response to TNF will not occur in the absence of transcription or translation inhibitors [30]. Inhibition of the anti-apoptotic signaling pathway by ligation of TNF-R via activation of TRAF2 and NF- $\kappa$ B is thought to be one of the mechanisms underlying the apoptosis-promoting effect of these inhibitors. Based on the above-mentioned results, we used TNF in combination with cycloheximide in all further incubations, unless otherwise stated.

### *Stimulation of p38 MAPK in TNF-induced apoptosis*

To ascertain whether p38 MAPK signaling is involved in TNF-mediated apoptosis, we used an anti-phospho-p38 MAPK Ab to detect activated p38 MAPK on Western blots. We found that treatment of EA.hy926 cells with TNF alone induced phosphorylation of p38 MAPK within 5 min (Fig. 2A), but the kinase was quickly de-phosphorylated

(within 30 min) in the absence of cycloheximide. By comparison, p38 MAPK phosphorylation was prolonged for several hours in cells co-treated with TNF and cycloheximide (Fig. 2B). Cycloheximide alone did not have any stimulatory effect on p38 MAPK (Fig. 2C).

#### Dose-dependent activation of p38 MAPK and cell death by TNF

To further investigate the possible involvement of p38 MAPK in TNF-induced cell death, we performed experiments with increasing concentrations of TNF and studied the effects on phosphorylation of p38 MAPK and cell death. The results show that the dose response curves for both effects were similar, and that 5 ng/ml TNF induced a clear increase in both p38 MAPK activity and cell death (mea-

sured as trypan blue permeable cells). The maximum responses were achieved with 10 ng/ml TNF (Figs. 3A and B).

#### SB203580 protects EA.hy926 cells from TNF-induced apoptosis

In light of our findings that treatment with TNF led to both phosphorylation of p38 MAPK, activation of caspase-3, and cell death, it seemed reasonable to assume that the p38 MAPK signaling is involved in TNF-induced apoptosis. To confirm that assumption, we tested the effects of the p38 MAPK inhibitor SB203580 on cell death and activation of caspase-3 in endothelial cells. We found that SB203580 conferred a significant (approximately 50%) decrease in the generated cell death and stimulation of caspase-3, detected as trypan blue permeability and cleav-

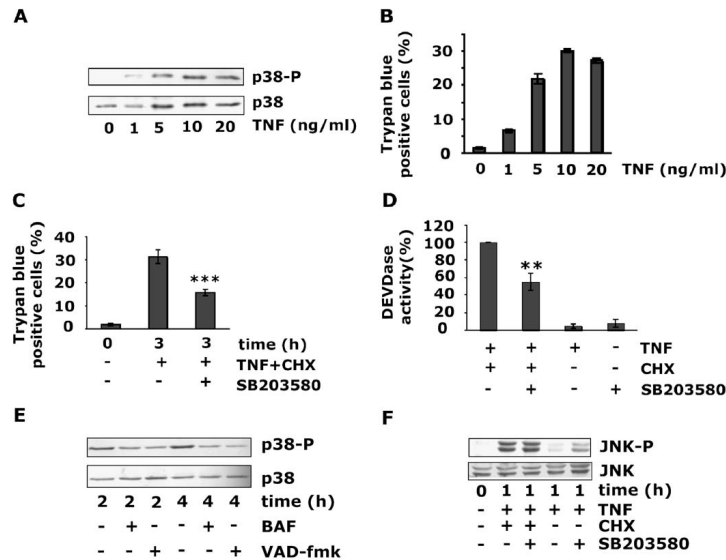


Fig. 3. Importance of p38 MAPK for cell death and caspase-3 activity. (A) EA.hy926 cells were exposed to increasing concentrations of TNF in combination with CHX (1  $\mu$ g/ml) for 3 h, and then processed for Western blotting. An anti-phospho-p38 MAPK Ab was used to detect the phosphorylated form of p38 MAPK (p38-P) on Western blots, after which the PVDF membrane was stripped and reprobed with an Ab against total cellular p38 MAPK as a loading control. The illustrated blot is representative of three independent experiments. (B) Cells were treated as in A and harvested for trypan blue assay as described in Materials and methods. The illustrated data represent means  $\pm$  SEM for six separate experiments. (C) Cells were exposed to TNF and CHX, and some dishes of cells were pretreated with SB203580 (20  $\mu$ M) for 30 min. After treatment, trypan blue permeability was analyzed, and the illustrated data represent means  $\pm$  SEM for five separate experiments. (D) Cells were exposed to TNF with or without CHX for 4 h, and some dishes of cells were pretreated with SB203580 for 30 min. After treatment, caspase-3 activity was determined as in Fig. 1; the level of activity induced by TNF + CHX was defined as 100%, and the other values were calculated in relation to this. The values shown are the mean  $\pm$  SEM of seven independent experiments. Statistical significance was determined by Student's two-tailed, unpaired *t* test; \*\**P*  $\leq$  0.001, \*\*\**P*  $\leq$  0.0001 compared to TNF + CHX. (E) Cells were or were not pretreated with a caspase inhibitor, either BAF (50  $\mu$ M) or VAD-fmk (20  $\mu$ M), for 30 min before the addition of TNF and CHX. An anti-phospho-p38 MAPK Ab was used to detect the phosphorylated form of p38 MAPK (p38-P) on Western blots, after which the PVDF membrane was stripped and reprobed with an Ab against total cellular p38 MAPK as a loading control. (F) Cells were exposed to TNF with or without CHX for 1 h, and in some cases the cells were preincubated with SB203580 for 30 min. Thereafter, the cells were lysed and processed for Western blotting, and JNK phosphorylation was assessed with an anti-phospho-JNK Ab (JNK-P). The results observed after incubation for 2 and 4 h were similar to those shown here. The PVDF membrane was subsequently stripped and reprobed with an anti-JNK Ab as a loading control (JNK). The illustrated blots are representative of four separate experiments.

age of DEVD-amc, respectively (Figs. 3C and D), which suggest that p38 MAPK activity is required for TNF-provoked apoptosis in endothelial cells. SB203580 or TNF alone had no significant impact on the level of cell death (data not shown) or caspase-3 activity (Fig. 3D).

To determine whether caspases participate in the regulation of p38 MAPK activity, we preincubated EA.hy926 cells with two different general caspase inhibitors, BAF and VAD-fmk, and then exposed them to TNF. As expected, Western blots probed with anti-phospho-p38 MAPK Ab showed that these two inhibitors did not affect early phosphorylation of p38 MAPK (i.e., phosphorylation seen during the first hour of TNF treatment; data not shown), whereas the later phase of p38 MAPK stimulation (i.e., after 2–4 h of TNF) was dependent on caspase activity (Fig. 3E).

Both p38 MAPK and JNK belong to the family of stress-activated protein kinases and have been shown to signal along similar pathways [32]. In agreement with results reported by other investigators [33], we found that TNF increased the phosphorylation or activation of JNK in endothelial cells, and that effect was potentiated by cycloheximide (Fig. 3E). SB203580 did not, however, inhibit activation of JNK. On the contrary, we observed a slight SB203580-induced stimulation of JNK phosphorylation after 1 h of exposure to TNF alone (Fig. 3F), and this effect persisted for up to 4 h of TNF treatment (data not shown).

#### Downregulation of cFLIP is not affected by SB203580 treatment

Other investigators [19,31] have suggested that cycloheximide-induced sensitization to death-receptor-mediated apoptosis may be due to downregulation of the cFLIP protein. Therefore, we examined the impact of TNF on the levels of two different splice forms of cFLIP, designated cFLIP<sub>L</sub> and cFLIP<sub>S</sub>. In agreement with previous studies, we found that treatment of EA.hy926 cells with TNF alone markedly increased the amounts of cFLIP after 2 h, whereas co-treatment with cycloheximide prevented this elevation, and in fact reduced the basal levels of both cFLIP<sub>L</sub> and cFLIP<sub>S</sub> after only 1 h (Fig. 4A).

To find out whether p38 MAPK affects downregulation of cFLIP, we preincubated EA.hy926 cells with SB203580, and then exposed them to TNF and cycloheximide. The results show that SB203580 did not increase cFLIP levels (Fig. 4B), and thus suggest that p38 MAPK is not involved in cFLIP downregulation during TNF-induced apoptosis.

#### p38 MAPK affects TNF-induced caspase-8 activity and Bid cleavage

We performed Western blot analysis to study the kinetics of TNF-induced activation of caspase-8 and found that the p18 active fragment of this enzyme was detectable after 2 h of treatment with TNF (Fig. 5A). Thus, the kinetics of cFLIP downregulation and caspase-8 activation support the

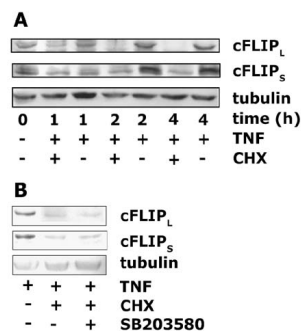


Fig. 4. Regulation of cFLIP protein levels in TNF-treated EA.hy926 cells. Cells were treated and processed as in Fig. 3, and levels of cFLIP<sub>L</sub> (55 kDa) and cFLIP<sub>S</sub> (25 kDa) were detected with an anti-cFLIP mAb that recognizes both these splice forms. PVDF membranes were subsequently stripped and reprobed with an anti- $\alpha$ -tubulin Ab as a loading control. The illustrated blots are representative of three independent experiments.

notion that cFLIP constrains caspase-8. Surprisingly, treatment with SB203580 reduced the activation of caspase-8 (Fig. 5A), which suggests that p38 MAPK could promote apoptosis at a site that is further upstream than previously presumed. However, it has been proposed that caspase-8 may also be stimulated by caspase-3 in a positive feedback loop that occurs via the mitochondria [34]. Accordingly, we conducted experiments using DQMD-fmk, which is a peptide that specifically inhibits caspase-3 and -6 and should therefore not have a direct influence on caspase-8. We found that DQMD-fmk prevented a major part of the proteolytic activation of caspase-8 (Fig. 5B), which indicates that this caspase was indeed stimulated by caspase-3 in our experimental system.

To further characterize the involvement of p38 MAPK in TNF-induced cell death, we studied the pro-apoptotic, BH3-domain-only protein Bid, which is an important substrate for caspase-8. It is believed that truncated Bid (tBid) mediates cell death via oligomerization of Bax or Bak with subsequent release of cytochrome *c* from the mitochondria [35]. We observed a time-dependent decrease in the 22-kDa band detected by the anti-Bid Ab on Western blots of lysates of TNF-treated EA.hy926 cells (Fig. 5C). This Ab recognizes only intact Bid in our experimental system, thus accumulation of tBid could not be seen on the blots. Cleavage of the 22-kDa Bid protein started at approximately the same time as caspase-8 activation, and we also noted that Bid cleavage was blocked in cells treated with SB203580 (Fig. 5C).

#### Increasing levels of activated p38 MAPK are found in mitochondria of endothelial cells exposed to TNF

In our search for mechanisms underlying the pro-apoptotic function of p38 MAPK, we next turned our attention to

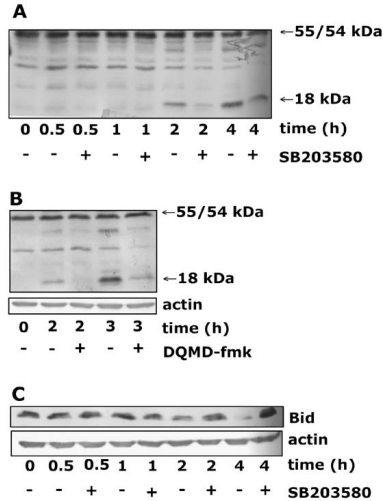


Fig. 5. Effects of p38 MAPK on caspase-8 activity and Bid cleavage in TNF-treated EA.hy926 cells. Cells were exposed to TNF and CHX for up to 4 h. In (A) and (B), some cells were preincubated in the absence or presence of SB203580 or 20  $\mu$ M DQMD-fmk (to inhibit caspase-3 and caspase-6) for 30 min. Thereafter, the cells were lysed and processed for Western blotting, and cleavage of procaspase-8 (55/54 kDa) was assessed with a mAb. The PVDF membranes were then stripped and reprobed with an anti-actin Ab as a loading control. In (C), some dishes of cells were pretreated with SB203580, after which they were lysed and processed for Western blotting. The cellular content of 22-kDa Bid was assessed using an anti-Bid Ab that does not recognize the proteolyzed form tBid in EA.hy926 cells. The membranes were then stripped and reprobed with an anti-actin Ab as a loading control. The blots shown are representative of three independent experiments.

the mitochondria, which constitute the center of control in most models of apoptosis [36]. The mitochondria are targeted by members of the Bcl-2 family, which include both anti- and pro-apoptotic proteins [24]. It is known that these proteins may be regulated by MAPK-effected phosphorylation at the mitochondria [37,38], thus we decided to look for active p38 MAPK in the mitochondrial subfraction of TNF-treated endothelial cells. In these experiments, we observed time-dependent, statistically significant accumulation of phosphorylated p38 MAPK in both the mitochondrial subfraction and the cytosol of TNF-treated cells (Fig. 6). The summarized data ( $n = 5$ ; Fig. 6B) indicate a 60% increase in phosphorylated mitochondrial p38 MAPK after 2 h of exposure to TNF and a 95% increase after 4 h of treatment.

*TNF induces a p38 MAPK- and proteasome-dependent decrease in the anti-apoptotic protein Bcl-x<sub>L</sub>*

To ascertain whether the apoptotic signaling of p38 MAPK involves members of the Bcl-2 family, we first

analyzed the levels of Bcl-2 proteins in TNF-treated endothelial cells. In the case of anti-apoptotic members of this family, our Western blotting results revealed that EA.hy926 cells did not contain any detectable amounts of Mcl-1 protein (data not shown). Bcl-2 and A1, on the other hand, were expressed by these cells, but the levels of these two proteins were not affected by treatment with TNF and cycloheximide in the presence or absence of SB203580 (Fig. 7A), or by exposure to TNF alone (data not shown). More importantly, we found that Bcl-x<sub>L</sub> was expressed at a moderate level, and TNF treatment induced an approximately 30% reduction in this protein (Figs. 7B and C). This decrease was observed only in the presence of cycloheximide, which agrees with the finding that cycloheximide is required for induction of apoptosis (Fig. 7C). Notably, pretreatment with SB203580 prevented the TNF-induced decrease in Bcl-x<sub>L</sub> (Figs. 7B and C). These results suggest that p38 MAPK mediates TNF-induced apoptosis of endothelial cells via modulation of the level of Bcl-x<sub>L</sub>.

We also incubated cells with lactacystin to discover whether the proteasomal system was responsible for the decrease in levels of Bcl-x<sub>L</sub> observed upon ligation of TNF-R. As shown in Figs. 7B and C, lactacystin prevented the TNF-induced downregulation of Bcl-x<sub>L</sub>, which suggests that proteasomal activity did cause the degradation of this protein. Furthermore, similar to the effect of SB203580, lactacystin effectively inhibited TNF-induced caspase-3

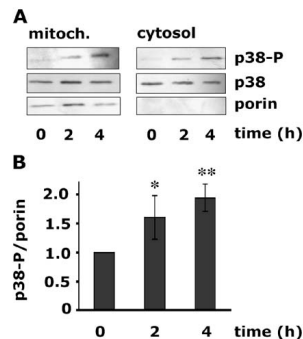


Fig. 6. Increase of phosphorylated p38 MAPK in the mitochondria of EA.hy926 cells. The cells were exposed to TNF and CHX for the indicated times, after which subcellular fractions were prepared as described in Materials and methods. The mitochondrial and cytosolic subfractions were processed for Western blotting, and the blots were probed with anti-phospho-p38 Ab (p38-P). For mitochondrial fractions, the whole sample was loaded onto the gel, whereas only 1/5 of the cytosol volume was loaded. The blots were subsequently stripped and reprobed with Abs recognizing total p38 MAPK (p38) and porin (a mitochondrial marker protein). (A) A blot representative of five independent experiments. (B) Scanning of the optical density (OD) of phospho-p38 MAPK bands and porin bands on Western blots of mitochondrial subfractions. The illustrated data represent means of calculated ratios ( $OD_{p38-P}/OD_{porin}$ )  $\pm$  SEM for five separate experiments; \*\* $P \leq 0.001$ , \* $P \leq 0.05$ .



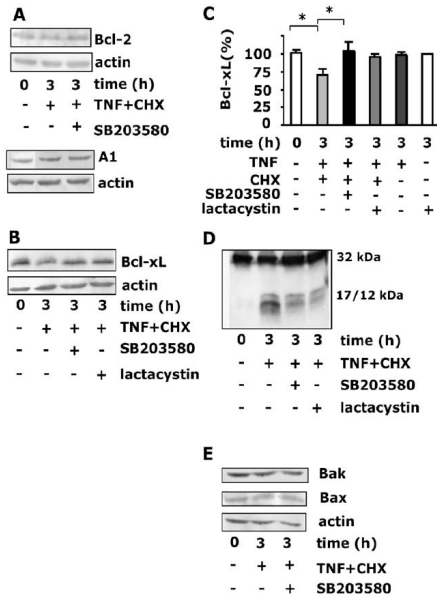


Fig. 7. Expression of Bcl-2 proteins in EA.hy926 cells. In (A) and (B), cells were exposed to TNF and CHX for 3 h, and some dishes of cells were preincubated with SB203580 for 30 min. After treatment, the cells were lysed and subjected to Western blotting, and the levels of Bcl-2, A1, and Bcl-xL were assessed with specific Abs. The membranes were subsequently reprobbed with an Ab against actin as a loading control. The blots shown are representative of three (A) and five (B) independent experiments. (C) The optical density (OD) of Bcl-xL bands and actin bands on Western blots was scanned, and the illustrated data represent the mean values of calculated ratios ( $OD_{Bcl-xL}/OD_{actin}$ )  $\pm$  SEM for five separate experiments; \* $P \leq 0.05$ . (D) Cells were treated as indicated and then processed for immunoblotting. Caspase-3 cleavage was detected with an Ab that recognizes intact procaspase-3 (32 kDa) and the active 17/12-kDa fragments. (E) The levels of Bak and Bax were assessed with specific Abs and thereafter, the membranes were reprobbed with an Ab against actin as a loading control. The blots shown are representative of three independent experiments.

activity (Fig. 7D). These results are compatible with the notion that proteasome-mediated Bcl-xL downregulation is an important step in TNF-induced apoptosis in endothelial cells.

#### Levels of Bax and Bak remain stable during TNF-induced apoptosis

The ratio between pro-apoptotic and anti-apoptotic members of the Bcl-2 family is critical in determining cellular susceptibility to apoptosis. Bax and Bak are the main pro-apoptotic Bcl-2 proteins that have been shown to exert their effects at the level of the mitochondria. We found that both Bax and Bak were expressed in EA.hy926 cells, and we also

noted that treatment with TNF, in the presence or absence of SB203580, did not change the levels of either of these proteins (Fig. 7E).

#### TNF causes p38 MAPK-dependent phosphorylation of Bcl-xL in endothelial cells

Because phosphorylation may cause a protein (in this case Bcl-xL) to be targeted for degradation in the proteasomes and p38 MAPK is a serine–threonine kinase, we decided to find out whether Bcl-xL can undergo TNF-induced serine–threonine phosphorylation. To achieve that goal, we immunoprecipitated Bcl-xL from TNF-treated endothelial cells and probed subsequent Western blots with a serine–threonine-specific Ab. The results show that TNF did indeed induce a serine–threonine phosphorylation of Bcl-xL (67.9% increase, Fig. 8B); moreover, this phos-

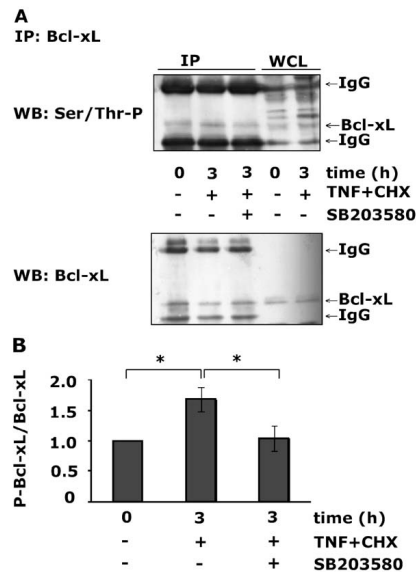


Fig. 8. p38 MAPK mediates phosphorylation of Bcl-xL in EA.hy926 cells treated with TNF. Cells were exposed to TNF and CHX for 3 h, and some were also pretreated with SB203580. After treatment, the cells were lysed, and Bcl-xL was immunoprecipitated as described in Materials and methods. (A) Cellular proteins were then separated by SDS-PAGE and immunoblotted. Western blots of the immunoprecipitates were probed with an anti-phospho-serine–threonine Ab and then stripped and reprobbed with an anti-Bcl-xL Ab. The blots shown are representative of four separate experiments. WCL, whole cell lysate. (B) The optical density (OD) of phospho-serine–threonine bands and Bcl-xL bands from immunoprecipitates was scanned, and the illustrated data represent the values of calculated ratios ( $OD_{P-Ser/Thr}/OD_{Bcl-xL}$ ) from four separate experiments.

phorylation was inhibited by SB203580 (Fig. 8). We also found that Bcl-x<sub>L</sub> seemed to be weakly phosphorylated in cells that had not been exposed to TNF, which might be explained by the small amount of active p38 MAPK that we detected in the mitochondria of the untreated cells (Fig. 6). We did not observe any co-immunoprecipitation of Bcl-x<sub>L</sub> and p38 MAPK after 3 h of incubation with TNF (data not shown), nonetheless the inhibitory effect of SB203580 on Bcl-x<sub>L</sub> phosphorylation suggests that p38 MAPK briefly associates with and phosphorylates Bcl-x<sub>L</sub> at some point in time.

## Discussion

It has become increasingly important to understand the mechanisms underlying induction of endothelial apoptosis because death of endothelial cells may limit unwanted neovascularization of tumors [39]. It has also been discovered that activation of apoptotic pathways in endothelial cells may be an important factor in atherosclerosis [40].

Our objective was to elucidate the mechanisms underlying p38 MAPK-mediated apoptosis in endothelial cells. We found that phosphorylation of p38 MAPK preceded the activation of caspase-3, and we also noted that prolonged phosphorylation of p38 MAPK appeared to be crucial for execution of the apoptotic program. It should be pointed out that, for up to 1 h of TNF treatment, stimulation of p38 MAPK did not depend on caspase activation. Furthermore, SB203580, which is an unusually specific inhibitor of p38 MAPK [41], caused approximately 50% inhibition of the TNF-induced activation of caspase-3 and cell death. However, at later time points (after 2–4 h of TNF treatment), caspase inhibitors did impede p38 MAPK phosphorylation, which implies the existence of caspase-induced feedback stimulation of p38 MAPK.

The question is, by what mechanism(s) does p38 MAPK mediate apoptosis in endothelial cells? It seems likely that p38 MAPK exerts its pro-apoptotic effect by phosphorylating substrate proteins, perhaps at different levels in the apoptosis cascade. Notwithstanding our finding that SB203580 treatment inhibited caspase-8 activity and Bid cleavage, it is plausible that p38 MAPK affects apoptosis at the mitochondrial level, since the activity of caspase-8 can also be amplified via the mitochondrial pathway. In support of the upstream involvement of p38 MAPK, Grambihler et al. [42] recently suggested that, in hepatocytes, p38 MAPK is associated with cFLIP<sub>L</sub> at the death-inducing signaling complex (DISC), and they also proposed that this association suppresses the activity of p38 MAPK.

We observed an increase in active p38 MAPK in the mitochondrial subfraction of TNF-treated cells, opening up for a possible p38 MAPK-induced phosphorylation of mitochondrial Bcl-2 proteins. Most importantly, we detected p38 MAPK-dependent serine–threonine phosphorylation and degradation of Bcl-x<sub>L</sub>, which suggests that this Bcl-2

protein is a major regulator of endothelial apoptosis. It has previously been shown that overexpression of Bcl-2 and Bcl-x<sub>L</sub> protects endothelial cells from TNF-induced apoptosis [43]. The reduction in Bcl-x<sub>L</sub> caused by TNF probably promotes apoptosis by triggering the release of pro-apoptotic members of the Bcl-2 family, proteins that normally form inactive heterodimers with anti-apoptotic Bcl-2 and Bcl-x<sub>L</sub>. In addition to demonstrating that caspase-3 mediates feedback stimulation of caspase-8, our findings provide an explanation for the absence of active caspase-8 fragments on Western blots during the first 2 h of TNF treatment, despite the earlier downregulation of cFLIP levels. The results of the co-immunoprecipitation experiments did not reveal any association between p38 MAPK and Bcl-x<sub>L</sub> in cells incubated with TNF for 3 h. Nevertheless, the inhibitory effect of SB203580 on phosphorylation of Bcl-x<sub>L</sub> suggests that, at some time during the initiation of apoptosis, p38 MAPK probably (at least briefly) joins with and phosphorylates Bcl-x<sub>L</sub>. The phosphorylation then seems to commit Bcl-x<sub>L</sub> to degradation in the proteasomal complexes, as indicated by our finding that lactacystin prevented the downregulation of Bcl-x<sub>L</sub> protein. It is known that the phosphorylation status of a protein is important in determining the stability of the molecule, and both phosphorylation and dephosphorylation have been reported to target proteins for degradation in proteasomes. In support of our results, Chadebech et al. [44] have observed phosphorylation and proteasome-mediated degradation of Bcl-2 in ovarian carcinoma cells treated with paclitaxel. Remarkably, we found that lactacystin also inhibited caspase-3 activity, which suggests that degradation of Bcl-x<sub>L</sub> is important for TNF-induced apoptosis in endothelial cells.

Similar to other investigators [45], we detected substantial amounts of Bcl-x<sub>L</sub> in the mitochondrial subfraction of EA.hy926 cells (data not shown), which is further evidence that p38 MAPK-mediated phosphorylation of Bcl-x<sub>L</sub> occurs at the level of the mitochondria. It is known that stress-activated protein kinases like JNK and p38 MAPK preferentially phosphorylate serine or threonine residues that are followed by prolines. These types of phosphorylation sites are found in Bcl-x<sub>L</sub> at Thr 47, Thr 115, and Ser 62. In a study by Kharbanda et al. [46], ionizing radiation was found to cause phosphorylation of Thr 47 and Thr 115 in Bcl-x<sub>L</sub> in U937 cells, and it was also noted that a mutant Bcl-x<sub>L</sub> with the threonines substituted by alanines was a more potent inhibitor of apoptosis. P38 MAPK-dependent phosphorylation of Bcl-x<sub>L</sub> was recently observed in prostate cancer cells [47], even though it is presumed that Bcl-x<sub>L</sub> phosphorylation promotes cell survival in that particular model. It has also been reported that p38 MAPK phosphorylates Bcl-2 and induces apoptosis in B-lymphocytes in response to NGF withdrawal [38]. Taxol is a well-known inducer of apoptosis and phosphorylation of Bcl-2 and Bcl-x<sub>L</sub> [48,49], and it has been suggested that such phosphorylation is mediated by JNK in prostate cancer cells [49]. We cannot rule out the possibility that JNK is involved in TNF-

induced endothelial apoptosis. However, our findings that SB203580 provided substantial protection against TNF-provoked death of EA.hy926 cells, whereas it did not inhibit the activity of JNK, suggest that JNK does not play a major role in this type of apoptosis.

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