Inhibition of Phosphatidylinositol 3-Kinase Sensitizes Vascular Endothelial Cells to Cytokine-initiated Cathepsin-dependent Apoptosis*

Lisa A. Madge‡, Jie-Hui Li, Jaehyuk Choi§, and Jordan S. Pober†¶

From the Interdepartmental Program in Vascular Biology and Transplantation, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

Received for publication, December 17, 2002, and in revised form, March 26, 2003
Published, JBC Papers in Press, March 27, 2003, DOI 10.1074/jbc.M212837200

Vascular endothelial cells (EC)^ are a principal target of the pro-inflammatory cytokines TNF and IL-1. Effects on the endothelium that contribute to the inflammatory response are largely dependent on gene transcription resulting in the expression of proteins controlling vasoregulation, leukocyte adhesion, leukocyte activation, and coagulation. In some cases TNF and IL-1 may also result in endothelial injury, a common feature in the pathogenesis of vascular leak, sepsis, and transplant rejection. The ability of TNF or IL-1 to cause endothelial injury may occur indirectly through the activation and recruitment of leukocytes or generation of thrombosis or may occur directly from the pro-apoptotic actions of these cytokines on EC.

The direct pro-apoptotic action of TNF on various cell types generally results from the ligand-dependent assembly of a death-inducing signaling complex (DISC), so called for the ability of this complex to initiate caspase activation. For TNF, the formation of a DISC is dependent on ligand-binding to TNF receptor type I (TNFR-1, also designated CD120a), which leads to the recruitment of the cytosolic adapter protein TNFR-1 associated death domain protein (TRADD)^ (1). The association between TNFR1 and TRADD involves the "death domains" (DD) of these proteins. DDs are homologous regions of ~80 amino acids that mediate protein-protein interaction and are also found in other receptors such as Fas (CD95)^ (2). TRADD may subsequently recruit Fas-associated death domain protein (FADD) through DD interactions (3). FADD contains both a DD and a death effector domain, the latter of which can interact with either pro-caspase-8 (also known as FLICE) or with cellular FLICE inhibitory protein (c-FLIP) (4, 5). FADD-associated pro-caspase-8 undergoes autocatalytic activation by proteolysis, liberating the active enzyme from the pro-form (6). c-FLIP may inhibit this process.

Activated caspase-8 dissociates from the DISC and acts on various cytosolic substrates. For example, caspase-8 may proteolytically activate the effector caspase-3. Activated caspase-3, in turn, cleaves a variety of substrates, resulting in apoptotic cell death (7). Alternatively, caspase-8 may proteolytically activate a cytosolic protein called Bid (8). Bid is a pro-apoptotic Bcl-2 family member containing a single Bcl-2 homology (BH) domain designated BH3. Proteolytically activated forms of "BH3-only" family members, such as Bid and Bad, bind to mitochondrial-associated "BH1–3" or "multidomain" proteins, such as Bax and Bak, causing supramolecular openings of the outer mitochondrial membrane (9–12). These openings allow homology domain; FKHR, forkhead transcription factor; AMC, aminomethylcoumarin; DAPI, 4',6-diamidino-2-phenylindole HCl; HA, hemagglutinin; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; HUVEC, human umbilical vein endothelial cell; zVADfmk, N-benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; CA-074-Me, [L-3-trans(propylcarbamoyl)oxirane-2-carboxyl]-L-isoleucyl-L-proline methyl ester.

* This work was supported in part by National Institutes of Health (NIH) Grants HL-36003 and HL-62188. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Supported by an American Heart Association Scientist Development grant.
§ Supported by National Institutes of Health Training Grant GM07205.
¶ To whom correspondence should be addressed: Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, CT 06536-0812. Tel.: 203-737-2292; Fax: 203-737-2293; E-mail: Jordan.Pober@yale.edu.

This paper is available on line at http://www.jbc.org
the release of cytochrome c from the mitochondria (13). Within the cytosol, cytochrome c associates with Apaf-1, and this complex further recruits pro-caspase-9 forming an assembly sometimes called an apoptosome. The apoptosome mediates ATP-dependent autocatalytic processing of caspase-9 and activated caspase-9, like caspase-8, can catalyze the proteolytic activation of caspase-3 resulting in apoptotic cell death (14). Thus caspase-8-mediated cleavage of Bid activates an amplification pathway for mitochondrial-dependent activation of caspase-3. Mitochondrial openings may also release other proteins, such as apoptosis-inducing factor that can initiate caspase-independent cell death (15). The requirement for the Bid/cytochrome c/Apaf1 amplification pathway differs among various cell types and correlates with the extent to which active caspase-8 is generated by the DISC (8, 16). DISC activity may be positively regulated by the expression levels of FADD and pro-caspase-8 (17) or negatively regulated by the levels of c-FLIP (5).

IL-1, like TNF, initiates the activation of signal transduction cascades by the recruitment of adapter proteins to its receptor. In EC, IL-1 may also initiate apoptotic cell death. To date, the precise components of the IL-1 receptor-associated DISC have not been defined. A number of adapter proteins involved in IL-1 signal transduction, namely MyD88 and IRAK (18), contain DDs and are possible mediators of caspase recruitment and activation.

Most untransformed cell types are not sensitive to the pro-apoptotic actions of TNF or IL-1, unless mRNA translation or protein synthesis is blocked. This observation has been explained by the capacity of TNF to stimulate the activation of NFκB, resulting in the up-regulation of anti-apoptotic gene products such as c-FLIP, XIAP, c-IAP 1, and c-IAP 2 (19–21).
The expression levels of several anti-apoptotic genes, such as c-FLIP and IAP 1 are also regulated by the proteasome (22, 23).

In the presence of cycloheximide (CHX), levels of c-FLIP are rapidly diminished, favoring DISC-dependent activation of caspase-8 (19, 22). Reduction of c-FLIP by antisense oligonucleotides mimics the effect of CHX and similarly sensitizes cells to death (22).

Although apoptosis is generally associated with caspase activation, either through a DISC or through an apoptosome, caspase-independent cell death has been observed with the generation of apoptotic-like features in a variety of cell types (24–26). In many instances, these variant forms of apoptosis are mediated by non-caspase proteases such as the cysteine protease cathepsin family, calpains, serine proteases, or the proteasome complex. Of particular interest, the activation of cathepsin B has been shown to play a central role in the generation on TNF-mediated cell death in fibrosarcoma cells (25). Furthermore, TNF-mediated apoptosis has been shown to be strongly reduced in hepatocytes from cathepsin B-deficient mice (27). This pathway has not been described in a normal (untransformed) human cell type.

While most TNF activities on EC result in inflammation and/or apoptosis, we have shown in EC that TNF and IL-1 also activate the anti-apoptotic phosphatidylinositol 3-kinase/Akt pathway (28). PI3K converts plasma membrane phosphatidylinositol 4,5-bisphosphate into phosphatidylinositol 3,4,5-trisphosphate, catalyzing the recruitment of several enzymes, such as PI3K-dependent protein kinase and Akt to the plasma membrane. Akt is a serine/threonine kinase that exerts an anti-apoptotic action by the phosphorylation of a number of substrates containing the phosphorylation consensus RXRXX(S/T). Akt-mediated phosphorylation inactivates the pro-apoptotic Bcl-2 homologue Bad (29), the apoptosis-initiating enzyme caspase-9 (30), and the forkhead family transcription factor FKHLR1 (31), which mediates transcription of pro-apoptotic gene products. In some cells Akt may regulate the activity of NFκB either through direct phosphorylation and activation of IKKα (32) or through regulation of the transactivation capacity of Rel A (33). We have shown that activation of PI3K in EC does not contribute to the activation of NFκB or have any significant effect on NFκB-dependent inflammatory responses (28). Similarly, Akt is reported not to have any effect of NFκB activation in HeLa cells (34).

In the present study we have examined the role of PI3K and Akt activation in the regulation of apoptosis induced by TNF and IL-1. To do so, we either inhibited PI3K with LY294002 or inhibited Akt by retroviral transduction with an inactive (K179M), dominant-negative form of Akt. We report that inhibition of PI3K but not of Akt sensitizes EC to the apoptotic actions of TNF and IL-1 and that the cell death caused by this pathway could not be blocked by caspase inhibition with zVAD-fmk but instead appears to be mediated through cathepsin B.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human umbilical vein EC were isolated from discarded tissue in accordance with an approved protocol by Yale University Human Investigations Committee and serially cultured on gelatin (J. T. Baker Inc., Phillipsburg, NJ)-coated tissue culture plastic (Falcon, Lincoln Park, NJ) in Medium 199 (M199) supplemented with 20% fetal calf serum, 20 μg/ml 1-glutamine (all from Invitrogen, Grand Island, NY), 50 μg/ml EC growth factor (ECGF) (Collaborative Biomedical Products, Bedford, MA), 100 μg/ml porcine heparin (Sigma Chemical Co., St. Louis, MO), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). All experiments were performed using EC at passage 2 or 3. Such sub-cultured EC uniformly display CD31 and von Willebrand substrate-attached were similarly re-plated following harvesting with trypsin (Invitrogen).

**Materials**—Recombinant human TNF was purchased from R&D, and IL-1 was purchased from Peprotech Inc (Rocky Hill, NJ). LY294002, CHX, and Z-Arg-Arg-amidomethylcoumarin (Z-Arg-Arg-AMC) were purchased from Calbiochem (San Diego, CA). Propidium iodide, Hoechst reagent, 4 ,6-diamidino-2-phenylindole HCl (DAPI), JC-1, calcein-AM, and LysoTracker red were purchased from Molecular Probes (Eugene, OR). RNase A and digitonin were purchased from Sigma. The cathepsin B inhibitor CA-074-Me was purchased from Peptides International (Louisville, KY). Complete protease inhibitor mixture tablets and Pefabloc were purchased from Roche Applied Science (Indianapolis, IN). Mouse anti-FLIP antibody was a gift from Dr. Peter Krammer (DFKZ, Heidelberg, Germany). Rabbit anti-Bid antibody was purchased from BD Pharmingen (San Jose, CA). Mouse anti-cathepsin B was purchased from Oncogene Research Products (San Diego, CA). CaspaTag fluorescein broad range (VAD), caspase-3 (DEVD), caspase-8 (LETD), and caspase-9 (LEHD) activity assay kits were purchased from Serologicals (Norcross, GA). Mouse anti-Bax antibody was purchased from Transduction Laboratories (San Jose, CA). Rabbit anti-FKHR and phospho-FKHR antibodies were purchased from Cell Signaling (Beverly, MA). Mouse anti-hemagglutinin (HA) was purchased from Roche Applied Science (Indianapolis, IN). Horseradish peroxidase-conjugated secondary antibodies for Western blotting were purchased from Jackson ImmunoResearch (Westgrove, PA).

**Immunoblotting**—For immunoblots, each well of a six-well plate containing a confluent HUVEC monolayer was washed twice in ice-cold PBS and lysed by the addition of 100 μl of lysis buffer (50 mM Tris-Cl, pH 6.8, 150 mM NaCl, and 1% Triton X-100) supplemented with Pefabloc (1 mM) and complete protease inhibitor mixture. For the measurement of phospho-proteins, NaF (10 mM) and Na3VO4 (1 mM) were also included in the lysis buffer to reduce phosphatase activity. After 20 min on ice, lysates were harvested by scraping. Where indicated, detached EC were harvested by centrifugation, washed in PBS, and pooled with the lysate of the attached EC from the same sample. For each sample, an equal amount of protein was separated by SDS-PAGE (35) then transferred electrophoretically to a polyvinylidene difluoride membrane.
A. Caspase activation at 3 hours

![Graph showing caspase activity at 3 hours with controls and treatments](image1)

B. Caspase activation at 18 hours

![Graph showing caspase activity at 18 hours with controls and treatments](image2)

**Fig. 3.** Inhibition of protein synthesis or PI3K sensitizes EC to TNF- or IL-1-mediated caspase activation. EC were stimulated with TNF or IL-1 (10 ng/ml) either alone (shaded histogram) or in the presence of CHX (open histogram) for 3 h prior to the addition of FAM-LETD-fmk (for caspase-8), FAM-LEHD-fmk (for caspase-9), or FAM-DEVD-fmk (for caspase-3) for a further 1-h incubation. Floating and attached EC were subsequently harvested, pooled, washed, fixed, and analyzed for caspase activity by FACS (A). For caspase activity analysis following inhibition of PI3K, EC were stimulated overnight with TNF or IL-1 (10 ng/ml) following pretreatment with vehicle (shaded histogram) or LY294002 (50 μM) (open histogram). FAM-coupled substrate was added for a further 1 h before harvesting as above (B).

(Immobilon P, Millipore, Milford, MA) and immunoblotted with primary and horseradish peroxidase-conjugated secondary antibodies. Detection of the bound antibody by enhanced chemiluminescence was performed according to the manufacturer’s instructions (Pierce Chemical Co., Rockford, IL).

**Cell Cycle and Hypodiploid DNA Analysis**—EC grown to confluence on 12-well plates were treated as described in the text. At the indicated time after treatment, floating EC were collected and pooled with residual attached EC suspended by trypsin treatment. The pooled EC were washed once in PBS and fixed by resuspension in 70% ethanol for 15 min. After fixation, EC were washed once more in PBS before incubation in PBS containing propidium iodide (50 μg/ml) and RNase A (1 mg/ml) for 0.25–2 h. The DNA content of EC was then determined by FACS analysis using Cell Quest software (FACSort, BD Biosciences, San Jose, CA).

**DAPI Staining of EC**—EC were grown to confluence on gelatin-coated 12-well plates and treated as described in the text. After treatment, attached EC were harvested on trypsin and combined with floating EC harvested from the same sample. Cells were washed in PBS, and ~1 × 10^6 cells were adhered to a glass coverslip by spinning in a cyto-centrifuge (Shandon, Pittsburgh, PA). Slides were air-dried and dipped in a chamber containing MeOH and DAPI (1 μg/ml). After rinsing in PBS, a drop of Gel Mount (Biomeda Corp., Foster City, CA) and a coverslip was placed over the cells. Specimens were examined by immunofluorescence microscopy using a Nikon diaphot microscope with a 360-nm filter.

**Quantitation of EC Adherence and Replating**—To quantify the number of cells that remained adherent, EC plated on gelatin-coated 96-well plates were treated as indicated in the text. After experimental manipulation the medium was removed and cells were washed twice in PBS. The residual attached cells were fixed and stained by the addition of 70% ethanol containing 100 μg/ml Hoescht 33258 reagent (Molecular Probes, Eugene, OR) for 30 min at room temperature. Cells were again washed twice with PBS, and the residual fluorescence was re-
Cytokine-initiated Cathepsin-dependent Apoptosis

RESULTS

TNF and IL-1 Mediate Nuclear Condensation and EC Death under Conditions Where Either Protein Synthesis Is Inhibited or PI3K Activation Is Blocked—Many cell types are not sensitive to the pro-apoptotic actions of TNF or IL-1 unless RNA or protein synthesis is blocked. As previously described (37), treatment of EC with TNF or IL-1 (10 ng/ml) in the presence of CHX (2.5 μg/ml) resulted in cell detachment (not shown) and in nuclear condensation and EC death (Fig. 1, top panels, E and F). In the present investigation, we compared the effect of inhibition of PI3K using the pharmacological inhibitor LY294002 with the effects of inhibiting protein synthesis. At concentrations of LY294002 between 30 and 50 μM, which we established to be effective in the inhibition of TNF-induced phosphorylation of Akt, we observed that pretreatment with LY294002 sensitized EC to the pro-apoptotic action of TNF or IL-1, resulting in extensive cell detachment (data not shown). EC that remained substrate-attached were able to re-adhere to gelatin-coated tissue culture plastic with close to 100% efficiency, whereas EC that had detached could not be re-plated, confirming that adherent cells remain viable, whereas detached cells are not. DAPI staining of cytosin preparations from pools of detached and viable cells confirmed that dead cells show nuclear condensation and fragmentation indicative of apoptosis (Fig. 1, bottom panels, E and F).

Inhibition of Akt Does Not Account for Sensitization to Cytokine-mediated Apoptosis—To determine whether the sensitization to TNF- or IL-1-mediated cell death upon inhibition of PI3K with LY294002 was dependent upon inhibition of Akt, EC were subjected to retroviral transduction with a dominant-negative inactive kinase form of Akt (K179M Akt). The functional expression of K179M Akt was confirmed by the inhibition of the TNF-dependent phosphorylation of the forkhead transcription factor (FKHR) (Fig. 2A). Surprisingly, expression of K179M Akt had no effect on cell survival following treatment with TNF or IL-1. Similarly, transduction with K179M Akt did not result in potentiation of cell death observed in response to LY294002 either alone or in combination with cytokine treatment. These experiments suggest that inhibition of Akt is not sufficient to explain the death response observed in response to cytokine following inhibition of PI3K. K179M Akt expression did cause a significant increase in the death response observed following treatment with cytokine plus CHX (Fig. 2B), suggesting that active Akt can limit caspase-induced cell death. The remainder of this study focused on the effects of LY294002 treatment.

Caspase Activity Assays—For experimental manipulation EC were plated on 12-well plates and treated at confluency as indicated in the text. After described treatment the CaspaTag peptide (FAM-VAD-fmk for broad range caspase activity, FAM-LETD-fmk for caspase-8, FAM-LHED-fmk for caspase-9, or FAM-DEVDF-fmk for caspase-9) was added to each well and incubated a further 1 h according to the manufacturer’s instructions. Subsequent to incubation with the peptide, floating EC were harvested and combined with attached EC from the same well. Substrate-attached EC were harvested, washed in Hanks’ balanced salt solution, and re-seeded onto gelatin-coated plates. EC that remained substrate-attached were similarly re-plated following harvest with trypsin. Viability was assessed as a replicating efficiency 18 h later quantified by Hoechst staining as above.

Mitochondrial Membrane Potential (ΔΨ) Analysis—After experimental manipulation of EC seeded on 12-well plates, floating EC were harvested by centrifugation and combined with remaining substrate-attached EC harvested with trypsin. The pooled EC were washed 1× in PBS containing 1% bovine serum albumin before resuspension in 200 μl of PBS/reduced serum albumin containing JC-1 (10 μg/ml). After 15 min of incubation at 37 °C, EC were washed, re-suspended in PBS, and analyzed by FACS.

Retroviral Transduction—HA-tagged murine K179M Akt (a gift from Dr. W. Sessa, Yale University) was Topo-transfected using EcoRI and NotI into the retroviral LZRS expression vector (a gift from Dr. A. L. M. Bothwell, Yale University), and the construct was verified by sequencing. The caspase-resistant Bcl-2 retroviral construct (a gift from A. L. M. Bothwell) has been described previously (36). The amphotropic Phoenix packaging cell line was transfected with either the empty vector LZRS, LZRS-K179M Akt, or LZRS-Bcl2 using LipofectAMINE (Invitrogen) and selected for gene expression 24 h after transfection using EGF (25). Puromycin-resistant cells were used to derive conditioned medium to provide a retroviral stock for HUVEC transduction. For transduction of primary HUVECs, M199 containing ECGF was removed, and cells were washed and incubated 5–8 h with retroviral conditioned media containing Polybrene (8 μg/ml, Sigma). After incubation, retrovirus was removed and replaced with normal growth medium overnight. The transduction process was repeated a further three times with intermittent cell passage as required. Using this protocol the percentage of HUVECs expressing the transgene is routinely >95%.

Preparation of Cytosolic Extracts for the Analysis of Caspase Activity—Measurement of cytosolic caspase B was determined using methodology similar to Foghsgaard et al. (25). Endothelial cells were pretreated with LY294002 (50 μM) for 3 h in complete M199 prior to the addition of cytokine for a further 3 h. After treatment media were removed and cells were washed twice in PBS prior to the addition of extraction buffer (50 μM MgCl2, 250 mM sucrose, 20 mM Heps, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM Pefabloc, pH 7.5) and incubation on ice for 20 min. (The conditions that allow for the selective permeabilization of the plasma membrane by digitonin without perturbation of lysosomes were determined in preliminary experiments using EC pre-loaded both with calcein-AM and LysoTracker red.) After incubation, the cytosolic extract was collected. Samples were analyzed for caspase B either by Western blotting or with a caspase B activity assay.

Measurement of Caspase B Activity—A 50-μl volume of cytosolic extract was added to an equal volume of caspase reaction buffer (50 mM sodium acetate, 4 mM EDTA, 8 mM dithiothreitol, 1 mM Pefabloc, pH 6.0). Caspase B activity was measured by the addition of 20 μM Z-Arg-Arg-AMC (Calbiochem). Liberated AMC was measured (λex = 360 nm, λem = 460 nm) using a fluorescence plate reader immediately following the addition of the peptide substrate (T0) and following a 60 min incubation at 37 °C (T60). Activity was determined by subtracting the background activity at T60 from activity at T0 and correcting for the amount of protein in each sample.

LY294002 sensitization of EC to the pro-apoptotic action of TNF or IL-1 (Fig. 4, A). EC were treated with TNF or IL-1 (10 ng/ml) overnight either alone (MOCK) or in the presence of CHX (2.5 μg/ml) or following pretreatment with LY294002 (50 μM) (B). EC lysates were subjected to SDS-PAGE and analyzed for the expression of c-FLIP by immunoblotting.

FIG. 4. Inhibition of PI3K does not result in c-FLIP depletion. EC were treated with either CHX (2.5 μg/ml) or LY294002 (50 μM) for various time intervals up to 24 h. EC lysates were separated by SDS-PAGE and analyzed for the expression of c-FLIP (A). EC were treated with TNF or IL-1 (10 ng/ml) overnight either alone (MOCK) or in the presence of CHX (2.5 μg/ml) or following pretreatment with LY294002 (50 μM) (B). EC lysates were subjected to SDS-PAGE and analyzed for the expression of c-FLIP by immunoblotting.
Activation of Caspase-8, -9, and -3 following Inhibition of PI3K or Treatment with Cycloheximide—The most proximal caspase activated by formation of a DISC is caspase-8. Activated caspase-8 may directly activate caspase-3 or, via its actions on the mitochondria, generate the apoptosome and activate caspase-9 (7, 8). We analyzed the effects of cycloheximide and PI3K inhibition on TNF- or IL-1-mediated activation of caspase-8 and subsequent downstream caspases-9 and -3. Using a living cell, fluorometric caspase assay (38) we observed activation of caspases-8, -9, and -3 within 1 h of treatment with cytokine plus CHX, which increased to a maximal activity at around 6 h (Fig. 3A). We could not clearly discern by this method sequential activation of these caspases, although caspase-8 activation was slightly earlier than activation of caspase-9 or -3 (not shown). Activation of caspases-8, -9, and -3 could also be observed in response to cytokine following inhibition of PI3K. In this case, however, caspase activation was slower and less extensive. No activation was observed for the first 6 h, and peak activity occurred only after an overnight incubation of 18 h or more (Fig. 3B).

Treatment of EC with Cycloheximide but Not LY294002 Decreases the Expression of c-FLIP—Sensitization of EC to cytokine-mediated apoptosis by TNF or IL-1 has been reported to result from the inhibition of NFκB-dependent transcription of anti-apoptotic genes coupled with degradation of the apoptosis inhibitor protein c-FLIP (19, 22). We confirm that treatment of EC with CHX results in a rapid decrease in the expression of c-FLIP (Fig. 4A). However, in contrast to the effect observed with CHX, inhibition of PI3K by treatment with LY294002 had no effect on the expression of c-FLIP, nor did it prevent TNF- or IL-1-induced up-regulation of c-FLIP (Fig. 4B).

Caspase Activation Is Not Required for Cytokine-initiated Cell Death following Inhibition of PI3K—Many of the features of programmed cell death such as detachment, DNA degrada-
Cytokine-initiated Cathepsin-dependent Apoptosis

Fig. 5. Effect of caspase inhibition on loss of ΔΨ. EC were treated in the absence or presence of zVADfmk (25 μM) with TNF or IL-1 (10 ng/ml) either alone (shaded histogram), in combination with CHX (2.5 μg/ml, open histogram) (A), or following pretreatment with LY294002 (50 μM, open histogram) (B). Floating and attached EC were harvested, pooled, washed, and re-suspended in PBS containing JC-1 (10 μg/ml). After 15 min at 37 °C EC were washed again, and ΔΨ was measured by FACS.

Fig. 6. Effect of caspase inhibition on loss of ΔΨ. EC were treated in the absence or presence of zVADfmk (25 μM) with TNF or IL-1 (10 ng/ml) either alone (shaded histogram), in combination with CHX (2.5 μg/ml, open histogram) (A), or following pretreatment with LY294002 (50 μM, open histogram) (B). Floating and attached EC were harvested, pooled, washed, and re-suspended in PBS containing JC-1 (10 μg/ml). After 15 min at 37 °C EC were washed again, and ΔΨ was measured by FACS.

Fig. 7. Bcl2 transduction protects EC from TNF- and IL-1-induced cell death in the presence of LY294002 but not cycloheximide. EC were subjected to retroviral transduction with either empty vector LZRS or caspase-resistant Bcl-2. Stable transductants were treated with TNF or IL-1 (10 ng/ml) either alone, following pretreatment with LY294002 (50 μM), or in combination with cycloheximide (2.5 μg/ml). After treatment overnight, floating EC were removed by washing, and substrate-attached EC were quantified by Hoescht staining.

PI3K or treatment with CHX assessed by PI staining and FACS analysis (Fig. 5C). Nuclear morphology by DAPI staining confirmed that treatment with TNF or IL-1 plus LY294002 resulted in the generation of fragmented nuclei (Fig. 1, bottom panel, E and F). Caspase inhibition completely prevented nuclear condensation associated with TNF or IL-1 plus CHX (Fig. 1, top panels, H and I). However, in the presence of zVADfmk,
cytokine plus LY294002 still resulted in nuclear condensation but did not progress to fragmentation (Fig. 1, bottom panel, H and I). Treatment with TNF or IL-1 in the presence of CHX or following inhibition of PI3K resulted in loss of \( \Delta \Psi \) as observed by an increase in fluorescence (FL-1) with the dye JC-1 (Fig. 6A). Caspase inhibition with zVADfmk blocked loss of \( \Delta \Psi \) in response to cytokine plus CHX but had no effect on the loss of \( \Delta \Psi \) in response to cytokine following inhibition of PI3K (Fig. 6B). These data suggested that, in the presence of LY294002, TNF and IL-1 activated a mitochondrial death pathway that was not dependent upon caspase activation. However, in the absence of zVADfmk, caspase activation did occur and the activation of caspases altered the morphological and biochemical features of cell death.

**Bcl2 Expression Blocks Cytokine-initiated Cell Death following Inhibition of PI3K but Not Protein Synthesis**—To further examine the role of the mitochondria in the death pathways evoked by TNF or IL-1 following inhibition of PI3K or treatment with CHX, EC were subjected to retroviral transduction with a caspase-resistant form of Bcl-2. Four rounds of infection with either empty vector (LZRS) or LZRS-Bcl-2 resulted in over 95% expression of the transgene in EC as confirmed by intracellular FACS staining (described previously in Ref. 36). We observed that transduction with Bcl-2 protected EC from cytokine-initiated cell death and detachment following inhibition of PI3K but had no effect or actually increased the killing observed in the presence of CHX (Fig. 7). Similarly, Bcl-2 expression effectively blocked both the activation of caspase-8 and loss of \( \Delta \Psi \) observed following inhibition of PI3K but had no effect or actually increased the killing observed in the presence of CHX (Fig. 7, A and B). Together these results support the observation that caspase activation in response to cytokine treatment following inhibition of PI3K results from rather than causes alterations in the mitochondria. In contrast, cytokine plus CHX-induced cell death occurs through a typical Type I death pathway that is completely

**Fig. 8.** Bcl2 transduction protects EC from TNF- and IL-1-induced caspase-8 activation and loss of \( \Delta \Psi \) following inhibition of PI3K but not protein synthesis. LZRS (shaded histogram)- or Bcl2 (open histogram)-transduced EC were treated with TNF or IL-1 (10 ng/ml) either alone, following pretreatment with LY294002 (50 \( \mu \)M) for 3 h, or in combination with CHX (2.5 \( \mu \)g/ml). After incubation overnight the caspase-8 substrate FAM-LETD-fmk was added for 1 h further before floating, attached EC were harvested, and caspase activity was determined with FACS. The numbers in parentheses reflect the FL-1 mean for Bcl-2 – the FL-1 mean for LZRS (A). EC were treated as described for panel A before floating, and attached EC were harvested, washed, and resuspended in PBS containing JC-1 (10 \( \mu \)g/ml). After 15 min at 37 °C EC were washed again and \( \Delta \Psi \) was measured by FACS (B).
Changes in the mitochondria of EC in this pathway occur downstream of caspase-8 activation and are not required for cell death.

Cathepsin B Activation Is Required for Cytokine-initiated Death following Inhibition of PI3K—We explored the possibility that the non-caspase protease cathepsin B could be the signal upstream of the mitochondria to initiate caspase-independent cell death following inhibition of PI3K using the specific cathepsin B inhibitor CA-074-Me. CA-074Me (30 μM) effectively blocked all of the features of cell death in response to cytokine following inhibition of PI3K such as increased EC detachment, DNA fragmentation, caspase activity, and loss of ΔΨ (Figs. 9, A–C, and 10B). In contrast, inhibition of cathepsin B had little or no effect on the same parameters observed in response to cytokine in the presence of CHX (Figs. 9, A–C, and 10A). Direct evidence that the activation of cathepsin B occurred in response to cytokine treatment following inhibition of PI3K was provided by translocation of active cathepsin B to the cytosol. Following only 3 h of treatment with LY294002 with or without TNF (i.e. at an earlier time than we could detect caspase activation) we observed an increase in immunoreactive cathepsin B in the cytosol using Western blotting (Fig. 11A). The appearance of cytosolic cathepsin B was paralleled by an increase in the cytosolic activity of the enzyme that was completely blocked in the presence of CA-074-Me (30 μM) (Fig. 11B).

To identify the mechanism through which an increase in cathepsin B activity might effect mitochondria to result in a loss of ΔΨ and activation of downstream effector caspases, we examined the effect of zVADfmk on the cleavage of Bid observed in response to cytokine plus CHX or following PI3K inhibition. In the presence of CHX or following inhibition of PI3K, TNF, and IL-1 resulted in a clear decrease in the levels of the 22-kDa inactive form of Bid. Following caspase inhibition with zVADfmk, the decrease in p22 Bid was blocked in both cases (data not shown). Similarly, it was also possible to observe dimerization of Bax in response to cytokine following inhibition of PI3K, and this was also blocked by caspase inhibition despite continued cell death (data not shown). These data indicate that treatment with TNF or IL-1 following inhibition of PI3K results in the activation of cathepsin B and that this activation accounts for the loss of mitochondrial function in
a manner that is not dependent on the cleavage of Bid and dimerization of Bax.

DISCUSSION

As previously reported, we observed that EC could be sensitized to the pro-apoptotic actions of TNF and IL-1 by inhibition of protein synthesis with CHX and demonstrated that the cell death observed showed features typical of apoptosis such as DNA degradation, nuclear condensation, and fragmentation and loss of $\Delta\Psi$. Several lines of evidence support the conclusion that the cell death resulting from treatment of EC with TNF or IL-1 in the presence of CHX can be primarily attributed to the mitochondrial-dependent Type I pathway described by Scaffidi et al. (16). The activation of caspases was rapid (detected following only 1 h of treatment) and in agreement with other studies associated with depletion of the endogenous caspase-8 inhibitor c-FLIP (19, 22). Furthermore, despite depletion of the pro-form of Bid and loss of $\Delta\Psi$, the activation of the mitochondrial pathway was not essential for the progression of apoptosis in response to cytokine plus CHX as shown by the fact that overexpression of Bcl-2 had no effect or actually decreased cell survival. Bcl-2 expression also had no effect on the activation of caspase-8 indicating that this activation occurred upstream of the loss of mitochondrial function. As expected, blocking caspase activation with zVADfmk prevented all morphological and biochemical parameters of apoptosis. zVADfmk-protected cells remained viable as indicated by effective replating, perhaps the most stringent assay of cell survival.

EC could also be sensitized to the pro-apoptotic actions of TNF and IL-1 following inhibition of PI3K with LY294002. This death pathway occurred with features typical of apoptosis such as DNA degradation, and nuclear condensation and fragmentation. However, caspase activation was delayed and less complete, and death could be blocked by overexpression of Bcl-2. These data initially suggested a Type II amplification pathway of caspase-3 activation. However, nuclear condensation, loss of $\Delta\Psi$, and cell death were still observed during complete caspase inhibition by zVADfmk as detected in an activity assay. Moreover, although it did not prevent death, zVADfmk still caused inhibition of nuclear fragmentation and of DNA degradation. These data suggest that caspases were activated and contributed to some of the morphological and biochemical features of death but that caspases were not necessary for death to occur. It is, however, formally possible that some degree of caspase activation occurs in the presence of zVADfmk that is too slight to measure yet still contributes to cell death. Given the effectiveness of inhibition of cytokine plus CHX treated by zVADfmk, this appears highly unlikely.

Caspase-independent cell death has previously been observed in a variety of other cell types. Caspase inhibition has been ineffective in suppressing anti-CD2 or staurosporine-in-
Cytokine-initiated Cathepsin-dependent Apoptosis

**A**

Mock | LY
--- | ---
CTR | TNF
CTR | TNF

**B**

Cytosolic cathepsin B activity (pmol/mg protein)

- **CTR**
- **TNF**

Fig. 11. Inhibition of PI3K results in translocation and activation of cathepsin B. For the analysis of the translocation of cathepsin B, cytosolic extracts were prepared by digitonin extraction under conditions that cause selective permeabilization of the plasma membrane without perturbing lysosomes. EC were treated for 3 h with vehicle with or without TNF following pretreatment with vehicle with or without LY294002 (50 μM). Cytosolic extracts were analyzed for cathepsin B by Western blotting (A). Similar results were observed in two other experiments. For the analysis of cathepsin B activity, EC were treated as above either with or without CA-074-Me (30 μM) (B). Data are mean ± S.E. of three observations. Similar results were observed in one other experiment.

duced death of activated T lymphocytes (24), TNF- plus CHX-mediated death of NIH3T3 cells (26), or TNF-mediated cell death of WEHI-S fibrosarcoma cells (25). The activation of non-caspase proteases such as cathepsins, calpains, or granzymes is now emerging as an alternate means to induce cell death (39, 40). Cathepsins are lysosomal proteases primarily thought to be involved in the degradation of proteins within the lysosomal compartment. However, several studies have shown translocation of cathepsins from the lysosome to the cytosol during cell death indicating that cathepsins may be able to gain access to cytosolic proteins (41, 42). The implication of cathepsin B activation during TNF-mediated cell death in mouse hepatocytes and human fibrosarcoma cells in vitro (25, 27, 41) prompted us to consider the role of cathepsin B in TNF- and IL-1-mediated cell death observed following inhibition of PI3K in EC. We observed that inhibition of cathepsin B with CA-074-Me prevented loss of ΔΨ, caspase activation, DNA fragmentation, and cell detachment observed in response to cytokine treatment following inhibition of PI3K. This inhibition occurred without significant effect on the same parameters activated in response to cytokine plus CHX. We also observed translocation of cathepsin B to the cytosol following inhibition of PI3K prior to the activation of caspases. Together, these results indicate that cathepsin B plays a critical role in a caspase-independent, mitochondrial-dependent death pathway in cytokine-treated EC.

We do not know the mechanism for cathepsin B activation in cells treated with TNF or IL-1 plus LY294002. Cathepsin B was liberated from the lysosomes by the actions of LY294002 alone. This agent produced some degree of cell death, but the presence of TNF or IL-1 markedly enhances this effect. We do not know how either LY294002 or cytokine work in this system. Because EC could not be sensitized to cytokine-mediated cell death following inhibition of Akt with K179M Akt, it is unlikely that Akt is involved in this pathway. Guicciardi et al. (41) reported that activation of cathepsin B by TNF plus actinomycin D was likely to be caspase-dependent, because recombinant caspase-8 could cause the release of cathepsin B from purified lysosomes. Our data do not support this conclusion. We observed that loss of ΔΨ was not blocked by inhibition of caspases, that transduction of EC with Bcl-2 prevented cytokine-dependent caspase-8 activation, and that the treatment of EC with LY294002 either alone or in combination with cytokine treatment failed to decrease levels of c-FLIP. We therefore conclude that the TNF- or IL-1-mediated death pathway activated in EC following inhibition of PI3K results from cathepsin B activation in a caspase-independent manner. A recent report has shown that selective lysosomal permeabilization by TNF in hepatocytes is paralleled by sphingosine-mediated lysosomal permeabilization (42). Inhibition of PI3K may sensitize EC to generate sphingosine either by the activation of sphingomyelinase (43) or by de novo biosynthesis (44).

How cathepsin B may result in the activation of a mitochondrial death pathway generating a phenotype with partial nuclear condensation is also undefined. Guicciardi et al. (41) allude to the possibility that cathepsin B activation might result in activation of a mitochondrial death pathway by the cleavage and activation of a cytosolic protein such as Bid. Stoka et al. (45) have also demonstrated cleavage of recombinant mouse Bid following treatment with isolated lysosomes. In contrast to these reports, we observed that caspase inhibition was able to prevent Bid depletion that occurred in response to cytokine treatment either following inhibition of PI3K or protein synthesis. Caspase inhibition was also completely effective in preventing the dimerization of Bax. These observations indicate that Bid cleavage and Bax dimerization are most likely consequences of caspase activation downstream of the mitochondria and not required to initiate cell death. We also failed to detect any increase in p53 as a result of treatment with cytokine plus LY294002. Identification of cathepsin substrates that are pertinent to the caspase-independent death pathway will be an important direction for future studies.

Acknowledgments—We thank Louise Camera Benson, Gwendoline Davis, and Lisa Gras for excellent assistance in cell culture.

REFERENCES

1. Hsu, H., Xiong, J., and Goeddel, D. V. (1995) Cell 81, 495–504
2. Boldin, M. P., Mett, I. L., Varfolomeev, E. E., Chumakov, I., Shemer-Avni, Y., Camonis, J. H., and Wallach, D. (1995) J. Biol. Chem. 270, 887–891
3. Hsu, H., Shu, H. B., Pan, M. G., and Goeddel, D. V. (1996) Cell 84, 299–308
4. Boldin, M. P., Goncharov, T. M., Golstei, V. Y., and Wallach, D. (1996) Cell 85, 803–815
5. Irmler, M., Thome, M., Mahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J. L., Schrotter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L. E., and Tschopp, J. (1997) Nature 388, 196–195
6. Muris, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998) J. Biol. Chem. 273, 2926–2931
7. Stennicke, H. R., Jurgensemeier, J. M., Shin, H., Deveraux, Q., Wolf, B. B., Yang, X., Zhou, Q., Ellerby, H. M., Ellerby, L. M., Breeden, D., Green, D. R., Reed, J. C., Frohlich, C. J., and Salvesen, G. S. (1998) J. Biol. Chem. 273, 27084–27090
8. Scaffidi, C., Schmitz, I., Zha, J., Koromayer, S. J., Kramer, P. H., and Peter, M. E. (1999) J. Biol. Chem. 274, 25533–25538
9. Ottolino, S., Diaz, J. L., Horn, W., Chang, J., Wang, Y., Wilson, G., Chang, S., Weeks, S., Fritz, L. C., and Olterdorff, T. (1997) J. Biol. Chem. 272, 28886–28872
10. Kuwana, T., Smith, J. J., Muzio, M., Dixit, V., Newmeyer, D. D., and Kornbluth, S. (1998) J. Biol. Chem. 273, 16589–16594

L. A. Madge and J. S. Pober, unpublished observations.
Cytokine-initiated Cathepsin-dependent Apoptosis

11. Gross, A., Jockel, J., Wei, M. C., and Korsmeyer, S. J. (1998) EMBO J. 17, 3878–3885
12. Kuwana, T., Mackey, M. R., Perkins, G., Ellisman, M. H., Latterich, M., Schneider, R., Green, D. R., and Newmeyer, D. D. (2002) Cell 111, 331–342
13. Luo, X., Budhanojo, I., Zou, H., Slaghter, C., and Wang, X. (1998) Cell 94, 481–490
14. Martin, S. J. (2002) Cell 109, 3586–3595
15. Li, J. H., Kluger, M. S., Madge, L. A., Zheng, L., Bothwell, A. L., and Pober, J. S. (2002) Am. J. Pathol. 159, 2045–2054
16. Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Kreuz, S., Siegmund, D., Scheurich, P., and Wajant, H. (2001) Mol. Cell. Biol. 21, 1687–1695
17. Joza, N., Susin, S. A., Daug man, K., Alevizopoulos, K., and Tschopp, J. (2001) EMBO J. 17, 1675–1687
18. Miechau, O., Lens, S., Gaide, O., Alevizopoulos, K., and Tschopp, J. (2001) EMBO J. 20, 3964–3976
19. Li, J. H., Kluger, M. S., Madge, L. A., Zheng, L., Bothwell, A. L., and Pober, J. S. (2002) Am. J. Pathol. 159, 2045–2054
20. Micheau, O., Lens, S., Gaide, O., Alevizopoulos, K., and Tschopp, J. (2001) EMBO J. 20, 3964–3976
21. Martin, S. J. (2002) Cell 109, 793–796
22. Bannerman, D. D., Tupper, J. C., Ricketts, W. A., Bennett, C. F., Winn, R. K., and Harlan, J. M. (2001) J. Biol. Chem. 276, 14924–14932
23. Yang, Y., Fang, S., Li, F., Maslak, M., and Karin, M. (2000) Science 288, 874–877
24. Deas, O., Dumont, C., MacFarlane, M., Rouleau, M., Hebih, C., Harper, F., Hirsch, P., Charpentier, B., Cohen, G. M., and Senik, A. (1998) J. Immunol. 161, 3375–3383
25. Fuchsberger, L., Wisnig, D., Mauch, D., Lademann, U., Bartholom, L., Boes, M., Elling, F., Leist, M., and Jaattela, M. (2001) J. Cell Biol. 153, 999–1010
26. Luscher, S., Ussat, S., Scherer, G., Kabelitz, D., and Adam-Klages, S. (2000) J. Biol. Chem. 275, 24670–24678
27. Guiciardi, M. E., Miyoshi, H., Bronk, S. F., and Gores, G. J. (2001) Am. J. Pathol. 159, 2045–2054
28. Madge, L. A., and Pober, J. S. (2000) J. Biol. Chem. 275, 15458–15465
29. Bannerman, D. D., Tupper, J. C., Ricketts, W. A., Bennett, C. F., Winn, R. K., and Harlan, J. M. (2001) EMBO J. 20, 3964–3976
30. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) Science 282, 1318–1321
31. Brunet, A., Ronni, A., Zigmund, M. J., Lin, M. Z., Xu, P., Hu, L. S., Andersen, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1997) Cell 91, 231–241
32. Martin, S. J. (2002) Cell 109, 3586–3595
33. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) Cell 91, 231–241
34. Delhase, M., Li, N., and Karin, M. (2000) Nature 406, 367–368
35. Slagsvold, P. M., De Luca, L. G., Min, W., and Pober, J. S. (1996) J. Immunol. 157, 4665–4671
36. Zheng, L., Dengler, T. J., Kluger, M. S., Madge, L. A., Schechener, J. S., Maher, S. E., Pober, J. S., and Bothwell, A. L. (2000) J. Immunol. 164, 7356–7347
37. Mathiasen, P. A., Yu, G., Johnson, G. L., Lee, B. W., Dhawan, S., and Phelps, D. J. (2001) BioTechniques 31, 608–610
38. Mathiasen, P. A., Yu, G., Johnson, G. L., Lee, B. W., Dhawan, S., and Phelps, D. J. (2001) BioTechniques 31, 608–610
39. Johnson, D. E. (2000) Leukemia 14, 1695–1703
40. Mathiasen, P. A., Yu, G., Johnson, G. L., Lee, B. W., Dhawan, S., and Phelps, D. J. (2001) BioTechniques 31, 608–610
41. Guiciardi, M. E., Deussing, J., Miyoshi, H., Bronk, S. F., Vening, P. A., Peters, C., Kaufmann, S. H., and Gores, G. J. (2000) J. Clin. Invest. 106, 1127–1137
42. Luscher, S., Ussat, S., Scherer, G., Kabelitz, D., and Adam-Klages, S. (2000) J. Biol. Chem. 275, 24670–24678
43. Modur, V., Zimmerman, G. A., Prescott, S. M., and McIntyre, T. M. (1996) J. Biol. Chem. 271, 13084–13102
44. Xia, P., Gamble, J. R., Rye, K. A., Wang, L., Hii, C. S., Cockerill, P., Khew-Goodall, Y., Bert, A. G., Barter, P. J., and Vadas, M. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14196–14201
45. Stoka, V., Turk, B., Schendel, S. L., Kim, T. H., Cirman, T., Stupas, S. J., Ellerby, L. M., Breiden, D., Freeze, H., Abrahamsson, M., Bromme, D., Krajewski, S., Reed, J. C., Yin, X. M., Turk, V., and Salvesen, G. S. (2001) J. Biol. Chem. 276, 3149–3157
