Hepatocyte Growth Factor Protects against Hypoxia/Reoxygenation-induced Apoptosis in Endothelial Cells*

Received for publication, August 21, 2003, and in revised form, October 31, 2003 Published, JBC Papers in Press, November 18, 2003, DOI 10.1074/jbc.M309271200

Xue Wang‡, Yushen Zhou§, Hong Pyo Kim‡, Ruiping Song‡, Reza Zarnegar‡, Stefan W. Ryter‡, and Augustine M. K. Choi‡‡

From the ‡Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, †Center for Biotechnology and Bioengineering, and ‡Division of Cellular and Molecular Pathology, Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania 15213

Hypoxia/reoxygenation causes cellular injury and death associated with a number of pathophysiological conditions, including myocardial ischemia/reperfusion injury and stroke. The cell death pathways induced by hypoxia/reoxygenation and their underlying regulatory mechanisms remain poorly understood. Recent studies have shown that hypoxia/reoxygenation can induce Bax translocation and cytochrome c release. Using murine lung endothelial cells as a model, we found that the induction of apoptosis by hypoxia/reoxygenation involved the activation of both Bax-dependent and death receptor-mediated pathways. We demonstrated the activation of the death-inducing signal complex and Bid pathway after hypoxia/reoxygenation. Hepatocyte growth factor markedly inhibited hypoxia/reoxygenation-induced endothelial cell apoptosis. The cytoprotection afforded by hepatocyte growth factor was mediated in part by the stimulation of FLICE-like inhibiting protein expression, the attenuation of death-inducing signal complex formation, and the inhibition of Bid and Bax activation. Hepatocyte growth factor also prevented cell injury and death by increasing the expression of the antiapoptotic Bcl-X<sub>L</sub> protein. The inhibition of Bid/Bax-induced cell death by hepatocyte growth factor primarily involved p38 MAPK and in part Akt-dependent pathways but not ERK1/ERK2.

Ischemia/reperfusion (I/R)<sup>1</sup> causes cellular dysfunction and tissue damage by generating cytotoxic reactive oxygen species and recruiting inflammatory leukocytes. The cellular death that occurs after ischemic insult has long been associated with necrosis. However, recent reports have established apoptosis as a significant contributor to cell death after I/R. Hypoxia, or decreased oxygen tension (pO<sub>2</sub>), arises during a variety of pathophysiological states such as ischemia, respiratory diseases, and tumorigenesis. Cell death may occur during the hypoxic phase preceding reoxygenation, in which case early membrane damage leads to the deregulation of degradative systems (necrosis) as well as the activation of cell death programs (apoptosis). Sublethal hypoxia damage may sensitize cells to reoxygenation-induced cell injury. Of particular clinical interest, an additional loss of parenchymal cells occurs during reoxygenation. The mechanisms of hypoxia/reoxygenation (H/R)-induced cell injury and death are not well known.

Two main apoptotic pathways have been defined in endothelial cells, an extrinsic pathway mediated by death receptor family proteins (i.e. Fas) and an intrinsic pathway mediated by the mitochondria. The initiation of the apoptotic process of cell death by H/R involves several possibilities. The expression of Fas ligand (FasL) during H/R may trigger Fas-dependent death pathways (1, 2) involving the recruitment and activation of caspase-8 and subsequent activation of effector caspases. This death cascade may also be amplified by caspase-8-dependent Bid activation, leading to mitochondrial damage and release of apoptogenic factors. H/R may cause irreparable mitochondrial damage because mammalian cells depend on mitochondria for long term viability. In human fetal alveolar type II epithelial cells the antiapoptotic proto-oncogene Bcl-2 displayed maximum abundance in hypoxia and mild reoxygenation (3). Hypoxia also induced a time-dependent mitochondrial translocation of Bax, a proapoptotic Bcl-2 family member, with the subsequent release of cytochrome c (4). Bcl-2 and Bax reciprocally control apoptosis by respectively inhibiting or stimulating mitochondrial cytochrome c release. Cytosolic cytochrome c and Apaf-1 cooperatively activate initiator caspase-9 that triggers a caspase cascade leading to apoptosis (5).

Hepatocyte growth factor (HGF), originally identified as a potent mitogen for mature hepatocytes, exerts potent mitogenic, mitogenic, and morphogenic activities on a wide variety of cells by activating the receptor tyrosine kinase cMet. The role of HGF-cMet signaling in the antiapoptotic effects of HGF remains unclear. The binding of HGF to cMet activates specific signaling pathways, including phosphatidylinositol 3-kinase (PI3K)-dependent Akt phosphorylation (6). Akt, a crucial antiapoptotic signal transducer with pleiotropic downstream effects, can inhibit apoptosis through phosphorylation and inactivation of the proapoptotic protein, Bad (7). Furthermore, cMet can inhibit apoptosis by binding to and sequestering the death receptors (i.e. Fas) to prevent receptor oligomerization and clustering (8) or by interacting with Bag, an antiapoptotic protein (9). Hypoxic conditions enhanced HGF expression whereas cMet expression was inhibited in cultured rat islets.

* This work was supported by American Heart Association Award AHA 0335035N, National Institutes of Health Training Grant T-32 (to S. W. R.), and National Institutes of Health Grants R01-HL60234, R01-A142965, and R01-HL55330 (to A. M. K. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This advertisement may be indexed for publication.*

† To whom correspondence should be addressed: Division of Pulmonary, Allergy and Critical Care Medicine, Dept. of Medicine, University of Pittsburgh Medical Center, 3459 Fifth Ave., MUH NW628, Pittsburgh, PA 15213. Tel.: 412-692-2210; Fax: 412-692-2260; E-mail: choiam@msx.upmc.edu.

‡ The abbreviations used are: I/R, ischemia/reperfusion; H/R, hypoxia/reoxygenation; HGF, hepatocyte growth factor; PI, phosphatidylinositol; MLEC, murine lung endothelial cell; MAPK, mitogen-activated protein kinase; DISC, death-inducing signal complex; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; FLIP, Fas-associated death domain (FADD) interleukin-1 β-converting enzyme-like (FLICE) inhibitory protein; LDH, lactate dehydrogenase.

This paper is available on line at http://www.jbc.org
togen-activated protein kinases (MAPK) and by blocking the FLIP, an inhibitor of caspase-8, by phosphorylating p38 mitogen-activated protein kinases (MAPK) and by blocking the mitochondrial translocation of Bax. Hypoxia induces a time-dependent cleavage of Bid via a death-inducing signal complex (DISC) formation and activation of caspase-8. HGF protects against H/R-induced apoptosis by up-regulating the expression of FLICE-like inhibiting protein (FLIP), an inhibitor of caspase-8, by phosphorylating p38 mitogen-activated protein kinases (MAPK) and by blocking the mitochondrial translocation of Bax.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—Antibodies anti-Bax, anti-Bid, anti-Bak, anti-caspase-3, -8, and -9, anti-Bcl-2, anti-Bcl-X	extsubscript{L}, anti-Fas, and anti-FLIP were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-phospho-p38 and anti-phospho-Bad from Cell Signaling Technology, Inc. (Beverly, MA); recombinant human HGF from R&D Systems, Inc. (Minneapolis, MN); digoxin from Sigma; lactate dehydrogenase (LDH) assay kit from Roche Diagnostics Corp.; adenosine-Hu-FLIP and adenovirus-LacZ were supplied by the Center for Biotechnology & Bioengineering, University of Pittsburgh (www.vectorcore.pitt.edu). PD98059, wortmannin, and SB203580 were purchased from Calbiochem-Novabiochem Corp. All other chemicals were from Sigma.

**Isolation and Culture of MLEC**—The isolation of endothelial cells by an immunobead protocol has been reported elsewhere (13). Briefly, mouse lungs were digested in collagenase and filtered through 100-μm cell strainers, centrifuged, and washed twice with medium. Cell suspensions were incubated with a monoclonal antibody (rat anti-mouse) against platelet endothelial cell adhesion molecule-1 for 30 min at 4°C. The cells were washed twice with buffer to remove unbound antibody and were resuspended in a binding buffer containing washed magnetic beads coated with sheep anti-rat IgG. Attached cells were washed four to five times in cell culture medium and then were digested with trypsin/EDTA to detach the beads. Bead-free cells were centrifuged and resuspended for culture. After two passages the cells were incubated with fluorescent labeled diacetylated low density lipoprotein, which is taken up only by endothelial cells and macrophages and sorted to homogeneity by fluorescence-activated cell sorter.

**Cell Culture and Treatments**—The MLEC were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 1% penicillin/streptomycin, 3.3 mM of endothelial cell growth supplements in humidified incubators at 37°C. For adenoviral infections, the cells were prepared with adenovirus-Hu-FLIP and adenovirus-LacZ were supplied by the Center for Biotechnology & Bioengineering, University of Pittsburgh (www.vectorcore.pitt.edu). PD98059, wortmannin, and SB203580 were purchased from Calbiochem-Novabiochem Corp. All other chemicals were from Sigma.

**LDH Release Assay**—LDH release was measured using a commercially available assay (Cytotoxicity Detection Kit, Roche Molecular Biochemicals). After 10 min of culture medium, the cells were harvested by trypsinization and were transferred to new tubes and then centrifuged again at 14,000 × g at 4°C for 20 min. The resulting supernatants were removed, and the pellets were retained for Western blotting.

**Western Blot Analysis**—Proteins were isolated from the culture of MLEC with radiomunoprecipitation assay buffer (1× phosphate-buffered saline, 1% [v/v] Nonidet P-40, 0.5% [w/v] sodium deoxycholate, 0.1% [w/v] SDS, 0.1 mg/ml phenylmethylsulfonyl fluoride, 30 μM apoaequorin, and 1 mM sodium orthovanadate). For immunoprecipitation, 1 μg of anti-Fas antibody was added to 500 μg of total protein in 500 μl, rotated for 2 h at 4°C, and then incubated with 20 μl of beads (protein A-succrose, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for another 2 h, spun down at 500 × g, and washed three times with radioimmunoprecipitation assay buffer. Then, 20 μl of loading buffer (100 mM Tris-HCl, 4% SDS, 0.2% bromphenol blue, and 20% glycerol) was added. For SDS-PAGE, samples containing equal amounts of protein were boiled in the loading buffer and separated on SDS-PAGE, followed by transfer to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk and stained with the primary antibodies for 2 h at the optimal concentrations. After five washes in phosphate-buffered saline with 0.2% Tween 20, the horseradish peroxidase-conjugated secondary antibody was applied, and the blot was developed with enhanced chemiluminescence reagents (Amersham Biosciences).

**Statistical Analysis**—All values are expressed as means ± S.E. Statistical significance was determined by Student’s t test, and a value of p < 0.05 was considered significant.

**RESULTS**

**H/R Induces Cell Death and Activation of Caspases**—To investigate the effect of H/R on pulmonary cell death, we used LDH release as a measure of cell death. MLEC were exposed to hypoxia (95% N	extsubscript{2}, 5% CO	extsubscript{2}) for 24 h and then reoxygenated to normal culture conditions (normoxia, 95% air, 5% CO	extsubscript{2}) for the indicated periods of time. As shown in Fig. 2A, after 3 h of reoxygenation the cell death was significantly increased compared with the cells in normoxia. Next we tested the hypothesis that cell death induced by H/R occurred via apoptosis, which is a caspase-mediated event. Flow cytometric analysis with PI/annexin-V double staining revealed that, after 24 h of continuous hypoxia, followed by 24 h of reoxygenation, 10–15% of the MLEC cells were apoptotic (Fig. 1B). After 24 h of hypoxia, followed by 2 h of reoxygenation, caspase-9 and -3 were activated (Fig. 1C). Based on these results we concluded that H/R induced caspase activation resulting in a time-dependent apoptotic cell death in MLEC.

**H/R-induced Apoptosis Activates Both Bax and Bid**—To elucidate the pathways involved in H/R-induced apoptosis in MLEC, we measured the DISC formation and the expression of Bax and Bid in MLEC during H/R. The formation of the DISC, as represented by the association of caspase-8 with Fas, occurred as an early event in H/R-induced injury. DISC formation reached an apparent maximum immediately following the hypoxia treatment and decayed rapidly within 1 h of reoxygenation (Fig. 2A). As shown in Fig. 2, B and C, both Bax and Bid were detected following H/R. Bax levels were highest at 12–24 h of reoxygenation following 24 h of hypoxia. The p15-truncated Bid (activated form) could be detected after hypoxia and then increased during the reoxygenation phase in a time-dependent manner.

**Bcl-X	extsubscript{L} Was Down-regulated during Reoxygenation**—The antiapoptotic Bcl-2 family members Bcl-2 and Bcl-X	extsubscript{L} inhibit Bax- and/or Bid-induced apoptosis by blocking mitochondrial cytochrome c release that occurs in response to a variety of apoptotic stimuli (4, 14). We examined the protein levels of Bcl-2 and Bcl-X	extsubscript{L} during H/R in MLEC. Bcl-X	extsubscript{L} levels did not change during hypoxia, relative to normoxic controls, but were downregulated after 3 h of reoxygenation (Fig. 2D). In contrast, the expression levels of Bcl-2 (Fig. 2D), FLIP, and Bak (Fig. 2E) did not change during the entire H/R treatment. These results suggest that Bcl-X	extsubscript{L} but not Bcl-2 participates in the modulation of apoptotic MLEC injury and death during reoxygenation.
HGF Protects MLEC from H/R-induced Cell Death by Blocking Bax Translocation and Inhibiting Bid Activation—We examined the effect of HGF treatment (50 ng/ml) on H/R-induced apoptosis in MLEC. As shown in Fig. 3A, HGF treatment significantly protected (p < 0.01) MLEC from cell death induced by H/R.

Given that H/R induces MLEC apoptosis via both Bax- and Bid-dependent pathways and HGF significantly decreases H/R-induced cell death, we hypothesized that HGF might inhibit both apoptotic pathways. We found that HGF blocked the mitochondrial translocation of Bax (Fig. 3B) and totally inhibited Bid cleavage (Fig. 3C). This HGF-mediated blockage of Bax translocation was not the result of a decrease in the level of Bax protein following these treatments because analysis of these same cell lysates indicated no change in the overall protein levels of Bax (Fig. 3B).

HGF Inhibited Bid Cleavage by Decreasing DISC Formation and Up-regulating FLIP Expression—To investigate the molecular mechanisms involved in HGF-mediated inhibition of Bid cleavage, we tested the hypothesis that HGF may block the formation of the DISC. H/R enhances the expression of Fas ligand (1, 2), which may trigger the Fas-driven apoptotic pathway. We examined the status of apoptotic mediators starting with the most proximal events in the Fas-induced apoptotic pathway, the hallmark of which is DISC formation. Activation of Fas results in receptor oligomerization and the rapid recruitment of Fas-associated death domain and caspase-8 to the cytoplasmic death domain of Fas. We treated the cells with HGF (50 ng/ml) for various times in low serum media (0.5% fetal bovine serum). As shown in Fig. 4A, treatment with hypoxia resulted in the formation of the DISC. Recruitment of caspase-8 to Fas was detectable after hypoxia but not under conditions of HGF treatment (Fig. 4A).

Given that hypoxia activates the extrinsic apoptotic pathway and that DISC formation can be decreased by HGF, we hypothesized that HGF may inhibit caspase-8 activation by modulation of a caspase-8 inhibitor such as FLIP. Indeed, HGF induced the levels of FLIP protein after hypoxia and during the reoxygenation phase (Fig. 4B). To gain further insight into the role of FLIP in H/R, we applied exogenous human FLIP to MLEC by adenoviral infection using adenoviral LacZ as a control. After 2 days the transfected cells were treated with H/R and assayed for apoptosis. The expression of FLIP in transfected cells is shown in Fig. 5A. As shown in Fig. 5B, FLIP...
significantly inhibited cell death ($p < 0.05$) after hypoxia. The amount of living cells was significantly ($p < 0.01$) higher in the FLIP-infected cultures than in the LacZ-infected cultures after 3 h of reoxygenation.

**HGF Blocked Bax Translocation via the p38 MAPK Signaling Pathway**—MAPK are signaling intermediaries that allow eukaryotic cells to interpret and respond to multiple stimuli. The p38 MAPK pathway has been traditionally associated with the stress and immune responses and more recently with the regulation of apoptosis and some differentiation processes (15). HGF may activate p38 MAPK in different cell types (16, 17). To investigate the mechanisms of the blockage of Bax translocation by HGF, we used three inhibitors, PD98059, a selective MEK1 inhibitor; wortmannin, which acts on the ATP binding site of PI3K and blocks the Akt signaling pathway; and SB203580, a selective inhibitor of p38 MAPK. After culturing the MLEC for 12 h in low serum, the compounds were added and incubated for 2 h before HGF treatment. We found that HGF can promote the phosphorylation of p38 MAPK (Fig. 6A). HGF inhibited Bax translocation via activation of p38 MAPK because Bax translocation could be completely reactivated with the p38 MAPK inhibitor, SB203580 (Fig. 6B). The PI3K/Akt inhibitor, wortmannin, slightly reactivated HGF-inhibitable Bax translocation. Furthermore, HGF can block Bid cleavage (Fig. 6B). The MEK/ERK 1/2 signaling pathways were apparently not involved in the suppression of Bax translocation by HGF (Fig. 6B). SB203580 aggravated H/R-induced cell injury and prevented HGF from protecting against H/R-induced cell injury (Fig. 6C). The ability of HGF to protect against H/R-induced cell injury was also compromised by wortmannin; however, this compound did not modulate H/R-induced cell injury in the absence of HGF. These data suggest that p38 MAPK kinase plays a critical role in H/R-induced cell injury. PI3K/Akt may play a partial role in HGF-mediated protection.

**HGF Stabilizes Bcl-X$_L$ Protein Levels after Reoxygenation**—It is well known that Bcl-X$_L$ can block mitochondrial release of cytochrome $c$ induced by Bax or Bid. Here, Bcl-X$_L$ was down-regulated after 3 h of reoxygenation (Fig. 2D). After treatment of MLEC with 50 ng/ml HGF, Bcl-X$_L$ protein was stable during reoxygenation compared with the cells that did not receive HGF treatment (Fig. 6D). These results suggest that another mechanism of HGF-mediated cytoprotection in MLEC may involve enhanced Bcl-X$_L$ expression, which inhibits Bax- and Bid-induced apoptotic cell death despite the mitochondrial translocation of these species.

**DISCUSSION**

The lung serves as the portal for O$_2$ delivery to the body, and the physiological mechanisms it adopts in response to hypoxia regulate the quantity of O$_2$ that is delivered to other organs. Acute exposure to hypoxia leads to vasoconstriction of resistance-sized pulmonary arteries, increases in pulmonary arterial pressure, and a redistribution of blood flow from the basal to the apical portion of the lung (18). H/R, which generates cytotoxic reactive oxygen species and promotes the recruitment of inflammatory leukocytes, causes lung injury and cell death involving both necrotic and apoptotic events. Endothelial cells appear to be the first cell type injured by reactive oxygen species generated during I/R (19). The mechanisms of apoptotic cell death induced by H/R, however, are not well understood, especially in endothelial cells.

Consistent with previous studies (4), H/R treatments enhanced Bax translocation in MLEC without significantly changing total Bax protein levels (12). The modulation of Bax translocation during H/R is not understood. The Bax induced by H/R is subject to inhibition by Bcl-2 and/or Bcl-X$_L$, which block cytochrome $c$ release from the mitochondria (4). Recent studies show that the Bid-mediated mitochondrial pathway is
critical to ischemic neuronal apoptosis and focal cerebral ischemia in a murine model (20, 21). Here, we present evidence that the Bid-mediated apoptotic pathway was activated in MLEC during H/R, especially at early time points.

The precise mechanism by which Bid is proteolytically activated in the lung after hypoxia is unclear at the present time. Several intracellular molecules, including caspase-8, granzyme B, and caspase-3, can activate Bid. The most efficient mechanism for Bid cleavage in various cell types is the Fas/Fas-associated death domain/caspase-8 pathway, which also appears to be a major pathway for Bid cleavage in MLEC. An increased expression of Fas ligand was observed during H/R in other cell types (1, 2) and in MLEC during the present study. We observed DISC formation after hypoxia (Fig. 2A), which cleaves caspase-8 to an active form that subsequently cleaves Bid. The proteolysis of Bid by caspase-3 may represent an important feedback loop for the amplification of apoptotic cell death (21). After 6 h of reoxygenation the caspase-8 in the DISC was significantly decreased (Fig. 4) because a similar amount of the truncated p15 Bid could be detected at 6 and 12 h of reoxygenation (Fig. 2C) in MLEC. Therefore the role of caspase-3 as a direct activator of Bid in H/R may be considered but not at the early times of cell injury (22).

HGF is present in the bronchoalveolar lavage fluid of normal adult rats and is responsible for most of the mitogenic effects of

FIG. 3. HGF prevents H/R-induced apoptotic cell death in MLEC by inhibition of Bax translocation and Bid cleavage. MLEC, cultured to 90% confluence, were incubated in low serum media (0.5% fetal calf serum) for 12 h, and then 50 ng/ml human HGF was added to the cultures for 2 h before hypoxia treatment. Cells were exposed to hypoxia (95% N₂, 5% CO₂) for 24 h and then restored to normal culture conditions (95% air, 5% CO₂). At the indicated times, 200 μl of supernatant medium was removed for LDH assays as described under “Experimental Procedures” (A). The data represent an average of two independent experiments with each sample in triplicate (n = 3). Experimental points were compared with normoxic controls (95% air, 5% CO₂) using Student’s t test (*, p < 0.05; and **, p < 0.01). Mitochondrial membranes or total protein were isolated at indicated times and subjected to Western blot analysis to detect Bax (B) and the p21 and p15 forms of Bid (C). β-Actin was used as a standard for protein loading.

FIG. 4. HGF blocks the formation of DISC and up-regulates FLIP protein expression. MLEC, cultured to 90% confluence, were incubated in low serum media (0.5% fetal calf serum) for 12 h, and then 50 ng/ml human HGF was added to the cultures for 2 h before hypoxia treatment. Cells were exposed to hypoxia (95% N₂, 5% CO₂) for 24 h and then restored to normal culture conditions (95% air, 5% CO₂) for the indicated times. Cell lysates subjected to immunoprecipitation with Fas or total protein were subjected to Western blot analysis to detect caspase 8 (A) and FLIP (B), as indicated. β-Actin was used as a standard for protein loading.

FIG. 5. FLIP inhibits apoptotic MLEC death during hypoxia and reoxygenation. MLEC at 30% confluence were cultured (in duplicate) under serum free conditions in the presence of 10⁶ plaque-forming units/ml of adeno-FLIP and adeno-LacZ for 3 h and then restored to normal medium. The expression of FLIP in transfected cells was monitored by Western analysis (A). Two days later, cells were exposed to hypoxia (95% N₂, 5% CO₂) for 24 h and then restored to normal culture conditions (95% air, 5% CO₂). At the indicated times, 200 μl of supernatant medium was removed for LDH assays as described under “Experimental Procedures” (B). The data represent an average of two independent experiments with each sample in triplicate (n = 3). Data from adeno-FLIP-infected cells was compared with the control (adeno-LacZ)-infected cells using Student’s t test (*, p < 0.05; and **, p < 0.01).
lavage fluid on alveolar epithelial cells (23). HGF expression increased in whole lung in a rat model of I/R (24). In this model, whole lung HGF mRNA increased by 24 h after I/R, followed by an increase in whole lung HGF protein that peaked at day 3 after I/R. Administration of an anti-HGF antibody aggravated I/R lung injury and reduced postinjury DNA synthesis in the lung, suggesting that endogenous HGF plays an important role in the reparative response to lung injury (24). HGF has been reported to be a survival factor for endothelial cells (25) and protects against human endothelial cell death during H/R in vitro (12). We report here that HGF significantly prevents MLEC death during H/R (Fig. 3A).

Collectively, from the results of our study and previously published reports, it appears that HGF inhibits I/R- or H/R-induced apoptosis by inhibition of DISC formation, inhibition of Bax translocation, and enhancement of Bcl-2 and/or Bcl-XL expression.

HGF increased the expression of FLIP protein (Fig. 4B), which can inhibit MLEC death after hypoxia and after reoxygenation (Fig. 5B). FLIP is a cytoplasmic protein with sequence homology to caspase-8. FLIP is capable of binding to Fas-associated death domain yet cannot undergo cleavage to an active caspase because of a substitution of a tyrosine for an active site cysteine, thus preventing the initiation of the death pathway (26). Cells with high levels of FLIP relative to caspase-8 are generally resistant to apoptosis (27). Therefore, HGF inhibits Bid cleavage completely in MLEC during H/R (Fig. 3C). FLIP is regulated by the PI3K/Akt signaling pathway (28). Whereas the PI3K/Akt inhibitor wortmannin slightly reversed the effect of HGF on Bax activation (Fig. 6B), we did not detect a significant modulation of Akt activation in MLEC with HGF treatment (data not shown).

HGF inhibits Bax mitochondrial translocation induced by high D-glucose concentration in endothelial cells (29). Consistent with this result, we show here that HGF blocks Bax translocation in MLEC during H/R, but the mechanisms remain unclear. In the presence of HGF, the p38 MAPK inhibitor, SB203580, reactivated the mitochondrial translocation of Bax and abolished the cytoprotective effect of HGF. In the absence of HGF inhibition of p38 MAPK with SB203580 increased H/R-induced cell death. These results indicate that p38 MAPK plays a critical role in H/R-induced cellular apoptosis. Our data suggest that both Bid and Bax are involved in apoptotic cell death but with potentially different roles. The importance of Bid may increase during the hypoxic phase and at early reoxygenation times whereas Bax may be important at later reoxygenation times, and likely does not cause cell death during the hypoxic phase.

**Fig. 6.** HGF activates p38 MAPK to inhibit Bax translocation during H/R. MLEC, cultured to 90% confluence, were incubated in low serum media (0.5% fetal calf serum) for 12 h. Fifty nanograms of human HGF per milliliter were added to the cultures for 2 h before hypoxia treatment. Cells were exposed to hypoxia (95% N₂, 5% CO₂) for 24 h and then restored to normal culture conditions (95% air, 5% CO₂) for the indicated times. Cell lysates were subjected to Western blot analysis to detect total and phospho-p38 MAPK (A). B, forty micromolar PD98059 (PD), 150 nm wortmannin (WM), or 30 μM SB203580 (SB) was added to the medium for 2 h before treatment with 50 ng/ml HGF for an additional 2 h. Cells were then subjected to H/R treatment for the indicated times. Cell lysates or mitochondrial membranes were subjected to Western blot analysis to detect Bax and Bid (B). At the indicated times, 200 μl of supernatant medium was removed for LDH assays as described under “Experimental Procedures” (C). The data represent an average of two independent experiments with each sample in triplicate (n = 3). Experimental points were compared with normoxic controls (95% air, 5% CO₂) using Student’s t test (**, p < 0.05; and ***, p < 0.01). Lysates from cells treated with H/R in the absence or presence of HGF were also analyzed for the expression of Bcl-XL (D). β-Actin was used as a standard for protein loading (B and D).
The antiapoptotic molecules in the Bcl-2 family (i.e., Bcl-2, Bcl-X_l) may be down-regulated in several cell types during H/R (30). HGF significantly increased Bcl-2 in human endothelial cells (12) and increased Bcl-X_l in MLEC (Fig. 6D). HGF triggers the phosphorylation and inactivation of the proapoptotic Bad protein via the PI3K/Akt pathway and simultaneously up-regulates Bcl-X_l (31). HGF blocked the induction of apoptosis by various DNA-damaging agents in breast cancer cells by preventing down-regulation of Bcl-X_l (32). The modulation of Bcl-X_l by HGF potentially involves three possible pathways, PI3K-dependent inactivation of Bad, NF-kB activation (33), and up-regulation of Bcl-X_l through unknown mechanisms. Recently, HGF has been reported to activate the signal transduction pathway for up-regulation of Bcl-X_l but not the PI3K-dependent pathway (11, 34), which is consistent with the results reported here.

In summary, we have examined apoptotic cell death in primary cultures of MLEC exposed to H/R stress and found that both extrinsic (Fas/caspase-8/Bid) and intrinsic (Bax/mitochondrial) pathways are involved. HGF can inhibit apoptotic cell death by increasing FLIP, which antagonizes DISC formation, thereby inhibiting caspase-8 activation and Bid cleavage. HGF also blocks Bax mitochondrial translocation and enhances the expression of the antiapoptotic protein Bcl-X_l, which is down-regulated during reoxygenation.

REFERENCES
1. Vogt, M., Bauer, M. K., Ferrari, D., and Schulze-Osthoff, K. (1996) FEBS Lett. 429, 67–72
2. Jeremias, I., Kupatt, C., Martin-Villalba, A., Habazettl, H., Schenkel, J., Beekstegers, P., and Debatin, K. M. (2000) Circulation 102, 915–923
3. Haddad, J. J., and Land, S. C. (2000) Biochem. Biophys. Res. Commun. 271, 257–267
4. Suikumar, P., Dong, Z., Patel, Y., Hall, K., Hopfer, U., and Weinberg, J. M. (1998) Oncogene 17, 3401–3415
5. Cain, K., Bratton, S. B., Cohen, G. M. (2002) Biochimie (Paris) 84, 203–214
6. Xiao, G. H., Jeffers, M., Bellacosa, A., Matsushita, Y., Vande Woude, G. F., and Testa, J. R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 247–252
7. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotlieb, Y., and Greenberg, M. E. (1997) Cell 91, 231–241
8. Wang, X., DeFrances, M. C., Dui, Y., Peddiatikus, P., Johnson, C., Bell, A., Michalopoulos, G. K., and Zarnegar, R. (2002) Mol. Cell 9, 411–421
9. Bardelli, A., Longati, P., Albero, D., Goruppi, S., Schneider, C., Ponzetto, C., and Comoglio, P. M. (1996) EMBO J. 15, 6205–6212
10. Vasi, B., Reitz, P., Xu, G., Sharma, A., Bonner-Weir, S., and Weir, G. C. (2000) Diabetesologia 43, 768–772
11. Nakamura, T., Mizuno, S., Matsumoto, K., Sawa, Y., Matsuda, H., and Nakamura, T. (2000) J. Clin. Invest. 106, 1511–1519
12. Yamamoto, K., Morishita, R., Hayashi, S., Matsushita, H., Nakagami, H., Moritani, A., Matsumoto, K., Nakamura, T., Kanesa, Y., and Oghara, T. (2001) Hypertension 37, 1341–1348
13. Tang, Z. L., Wasserman, K., L., Xu, X., Stitt, M. S., Reynolds, I. J., Pitt, B. R., and St. Croix, C. M. (2002) Mol. Cell Biochem. 234–235, 211–217
14. Yang, J., Liu, X., Xuhla, K., Kim, C. N., Brade, A. M., Cai, J., Peng, T.-I., Jones, D. P., and Wang, X. (1997) Science 275, 1129–1132
15. Nebreda, A. R., and Ferras, A. (2000) Trends Biochem. Sci. 25, 257–260
16. Cartwright, J. E., Tse, W. K., and Whitley, G. H. (2002) Exp. Cell Res. 279, 219–226
17. Muller, M., Moretti, A., and Ponzetto, C. (2002) Mol. Cell Biol. 22, 1060–1072
18. Hansen, T. N., Le Blanc, A. L., and Gest, A. L. (1985) J. Appl. Physiol. 58, 812–818
19. Sunnergen, K. P., and Rovetto, M. J. (1997) Am. J. Physiol. 252, H1211–H1217
20. Plesilsa, N., Zinkel, S., Le, D. A., Amin-Hanjani, S., Wu, Y., Qiu, J., Chiarugi, A., Thomas, S. S., Kohane, D. S., Korsmeyer, S. J., and Moskowitz, M. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 98, 15318–15323
21. Siles, E. A. Keogh, S. A., and Martin, S. J. (2000) Cell Death Differ. 7, 556–565
22. Yin, X. M., Luo, Y., Cao, G., Bai, L., Pei, W., Kuharsky, D. K., and Chen, J. (2002) J. Biol. Chem. 277, 42074–42081
23. Mason, R. J., McCormick-Shannon, K., Rubin, J. S., Nakamura, T., and Leslie, C. C. (1996) Am. J. Physiol. 271, L146–L153
24. Yamada, T., Hisanaga, M., Nakajima, Y., Mizuno, S., Matsumoto, K., Nakamura, T., and Nakano, H. (2000) Am. J. Respir. Crit. Care Med. 162, 707–715
25. Ma, H., Calderon, T. M., Fallon, J. T., and Berman, J. W. (2002) Hypertension 39, 129–134
26. Bhowmick, N. A., Thomas, S. S., Kohane, D. S., Korsmeyer, S. J., and Moskowitz, M. A. (2000) Am. J. Physiol. 278, L46–L53
27. Hoffmann, A., and St. Croix, C. M. (2002) Mol. Cell Biochem. 234, 9–15
28. Yamada, T., Hisanaga, M., Nakajima, Y., Mizuno, S., Matsumoto, K., Nakamura, T., and Nakano, H. (2000) Am. J. Respir. Crit. Care Med. 162, 707–715
29. Saikumar, P., Dong, Z., Weinberg, J. M., and Comoglio, P. M. (1996) EMBO J. 15, 7293–7301
30. Saikumar, P., Dong, Z., Weinberg, J. M., and Venkatachalam, M. A. (1998) Oncogene 17, 3341–3349
31. Liu, Y. (1999) Am. J. Physiol. 277, F624–F633
32. Fan, S., Wang, J. A., Yuan, R. Q., Rockwell, S., Andrews, J., Zlatapolskiy, A., Goldberg, I. D., and Rosen, E. M. (1998) Oncogene 17, 131–141
33. Chen, F., Demers, L. M., Vallathavan, V., Lu, Y., Castranova, V., and Shi, X. (1999) J. Biol. Chem. 274, 35591–35595
34. Klett, K., Day, R. M., Kim, Y., Torreggiosi, I., Evans, T., and Suzuki, Y. J. (2003) J. Biol. Chem. 278, 4705–4712