Hypoxia sensitization of hepatocytes to neutrophil elastase-mediated cell death depends on MAPKs and HIF-1α

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Sparkenbaugh EM, Ganey PE, Roth RA. Hypoxia sensitization of hepatocytes to neutrophil elastase-mediated cell death depends on MAPKs and HIF-1α. Am J Physiol Gastrointest Liver Physiol 302: G748–G757, 2012. First published January 5, 2012; doi:10.1152/ajpgi.00409.2011.—The liver is sensitive to pathological conditions associated with tissue hypoxia (Hx) and the presence of activated neutrophils that secrete the serine protease elastase (EL). We demonstrated previously that cotreatment of rat hepatocytes with nontoxic levels of Hx and EL caused synergistic cell death. Hx is sensed by hypoxia-inducible factor (HIF)-1α, a transcription factor that heterodimerizes with HIF-1β, nuclear receptor nuclear translocator and directs expression of many genes, including the pro-cell death gene Bcl-2/adenovirus E1B-interacting protein 3 (BNIP3). Since cell death from EL or Hx also requires MAPK activation, we tested the hypothesis that the cytotoxic interaction of Hx and EL depends on MAPK and HIF-1α signaling. Treatment of Hepa1c1c7 cells with EL in the presence of Hx (2% O2), resulted in synergistic cell death. EL reduced phosphorylated ERK in O2-replete and Hx-exposed cells, and ERK inhibition enhanced the cytotoxicity of EL alone. Hx-EL cotreatment caused an additive increase in phosphorylated p38, and p38 phosphorylation attenuated EL cytotoxicity of EL by cotreatment with eglin C or genetic knockout of EL prevents liver injury in some models of inflammatory stress-drug interaction (7, 33). In vitro, exposure of primary rat HPCs to modest Hx (5% O2) enhanced the cytotoxicity of EL and shortened the time to onset of cell death (20), further indicating that there is an interaction in the pathway(s) by which Hx and EL cause injury. The mechanism of this interaction is not known.

EL is cytotoxic to many primary and transformed cell types in vitro, including lung epithelial cells (26, 35) and primary HPCs (10, 21); however, the mechanism is not well understood. In primary HPCs, EL induces morphological changes such as mitochondrial, endoplasmic reticular, and nuclear swelling, consistent with oncotic necrosis (21). In transformed lung epithelial cells, EL activates MAPKs and NF-κB in a protease-activated receptor-dependent fashion, resulting in MAPK- and NF-κB-dependent apoptosis (36, 37). Thus the mechanism of EL cytotoxicity might depend on cell type, but it is clear that EL can cause cell membrane dysfunction and activate cell death signaling pathways.

During inflammatory episodes, a reduction in tissue PO2 (i.e., Hx) can occur from decreased tissue perfusion as a result of vascular obstruction from coagulation-mediated fibrin deposition (20) or increased metabolic demand of innate immune cells (15). In addition, EL inhibits endothelial cell production of nitric oxide and prostacyclin, thereby inhibiting vasodilation and reducing blood flow to hepatic tissue, further contributing to the development of Hx (25).

Hypoxia-inducible factor (HIF) has been widely studied as a factor used by cells to sense and respond to changes in PO2. HIF comprises α- and β-subunits, which heterodimerize to form a competent transcription factor (30). There are three isoforms of the α-subunit, HIF-1α, HIF-2α, and HIF-3α, of which HIF-1α is the best characterized. The β-subunit, HIF-1β, is also known as the aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-1α is constitutively expressed in the cytoplasm, and under normal PO2, it is continually degraded. When O2 is limited, HIF-1α can accumulate and translocate to the nucleus, where it heterodimerizes with HIF-1β/ARNT, binds to hypoxia response elements in the DNA, and directs transcription of >100 genes (30). HIF-1α-directed genes control many pathways, including angiogenesis, erythropoiesis, energy metabolism, and glycolysis, all of which can enhance O2 delivery or energy production in Hx environments. However, if Hx is severe or prolonged, HIF-1α can also activate transcription of cell death genes, including Bcl-2/adenovirus E1B-interacting protein 3 (BNIP3) and Nix (18).

Hx injures HPCs through a variety of pathways in addition to HIF-1α. It causes mitochondrial dysfunction, loss of ATP production, and eventual loss of plasma membrane integrity (16). Exposure of primary HPCs or the transformed hepatocyte...
cell line HepG2 to low PO2 (<10 mmHg in the cell culture atmosphere) increases mitochondrial reactive oxygen species (ROS) production (2), which contributes to Hx-mediated cell death by damaging DNA, proteins, and polysaccharides and initiating lipid peroxidation (17). Recent studies indicate that the Hx phase of hypoxia-reoxygenation injury involves lipid peroxidation and membrane damage in mouse embryonic fibroblasts (41). Furthermore, Hx activates the MAPKs (31). In particular, activation of p38 MAPK enhances gene transcription by HIF-1α (23), as well as Hx-induced death of primary HPCs (22).

There is significant overlap in the mechanisms by which Hx and EL lead to HPC injury separately; however, the mechanism by which Hx sensitizes HPCs to killing by EL is not known. We hypothesized that the synergistic cytotoxicity of Hx and EL requires MAPKs and HIF-1α signaling. The results from these studies offer insight into mechanisms of HPC death that can lead to liver failure in situations in which tissue Hx and PMN-associated inflammation occur.

**EXPERIMENTAL PROCEDURES**

**Materials.** Unless otherwise noted, all materials were purchased from Sigma-Aldrich (St. Louis, MO). Antibiotic-antimycotic and DMEM were purchased from Invitrogen (Carlsbad, CA). Heat-inactivated FBS was purchased from Fisher Scientific. SB-203580, U-0126, SP-600125, elastase inhibitor III, and 2-(4-methyl-1-8-(morpholin-4-ylsulfonyl)-1,3-dioxo-1,3-dihydro-2H-pyrrolo[3,4-c]quinolin-2-yl)ethyl acetate, a nonpeptidyl pyrroloquinoline caspase inhibitor (CI), were purchased from Calbiochem (San Diego, CA).

**Elastase.** Human sputum elastase (Elastin Products, Owensville, MO) was resuspended in PBS and stored at −20°C until use. The enzymatic activity of EL was determined using the colorimetric elastase substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (Calbiochem). One unit of EL was defined as the amount of enzyme required to cause a change of absorbance of 1.0 at 410 nm in 10 min at 37°C.

**Hx exposures.** For Hx exposures, cells were placed in heated, humidified hypoxic glove boxes (Coy Laboratories, Grass Lake, MI) at 5% CO2. Primary HPCs were exposed to 5% O2, and Hepa1c1c7 cells were exposed to 2% O2 in the atmosphere. Chamber PO2 was monitored continually to ensure consistent O2 exposure.

**Cell culture.** Primary rat HPCs were isolated as previously described (20). Mouse Hepa1c1c7 and HepaC4 cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic at 20% O2 (5% CO2, 37°C) for 1 wk prior to experiments. HepaC4 cells are derived from Hepa1c1c7 cells; the HIF-1β deficiency results from point mutation in the HIF-1β gene due to selection of mutagen-exposed colonies that were unresponsive to HIF-1β inducers (American Type Culture Collection). Confluent cell cultures were detached from the plate with 0.25% trypsin-EDTA. For protein and RNA isolation, cells were seeded at 1 × 10^6 cells per well in 6-well tissue culture dishes (Costar, Lowell, MA), and for cytotoxicity experiments, cells were seeded at 4 × 10^5 cells per well in white-walled 96-well plates (Costar). Cells were allowed to adhere overnight at 37°C.

**Cytotoxicity assessment.** HPCs were plated at 2.5 × 10^4 cells per well in 12-well tissue culture plates in Williams’ medium E (Invitrogen) containing 10% FBS for 3 h. After attachment, the cells were washed twice with serum-free Williams’ medium E and treated with various concentrations of EL in O2-replete [OxR (20% O2 and 5% CO2, 37°C)] or a Hx environment for 8 h. At 8 h, medium and cell lysates were collected and analyzed for alanine aminotransferase activity, as described previously (20).

Hepa1c1c7 and HepaC4 cells were allowed to adhere and were washed with serum-free DMEM to remove traces of FBS, which can neutralize the proteolytic activity of EL. Cells were treated with various concentrations of EL in serum-free DMEM in O2-replete [OxR (20% O2 and 5% CO2, 37°C)] or in medium that had been incubated overnight in a hypoxic atmosphere (primary HPCs at 5% O2, Hepa1c1c7 and HepaC4 cells at 2% or 5% O2, 37°C) and placed in the OxR or Hx incubator. After treatment, the pericellular PO2 was monitored fluorometrically using OxoDishes and the Sensor Dish Reader (PreSens, Regensburg, Germany). The pericellular PO2 adjacent to OxR and Hx cells reached a steady state of 140 and 10 mmHg, respectively, within 3 h.

Primary HPCs were exposed to 5% O2 for Hx, which was a level of Hx that did not cause injury on its own but did interact with EL. The Hepa1c1c7 and HepaC4 cells were exposed to 5%, 2%, and 1% O2 with EL. There was no interaction at 5% O2, and 1% O2 caused significant cytotoxicity. There was a consistent, robust interaction at 2% O2, and this Hx condition was used for subsequent studies. For experiments using Hepa1c1c7 and HepaC4 cells, cytotoxicity was assessed with the CytoTox-Glo luminescence assay (Promega, Madison, WI) according to the manufacturer’s directions. Briefly, the CytoTox-Glo luminescence assay measures the activity of an intracellular protease that has been released from membrane-compromised cells. The dead cell protease activity is measured in the medium and in the cell lysate, and data are represented as percentage of total cell protease released (percent enzyme release).

For MAPK inhibitor studies, stock solutions of SB-203580, U-0126, and SP-600125 were prepared in DMSO and stored at −70°C. Cells were washed twice with serum-free DMEM and treated with 5 or 10 µM inhibitor (0.1% DMSO vehicle) for 2 h in an OxR incubator. For CI studies, a stock solution of CI was prepared in DMEM and stored at −20°C. Cells were washed twice with serum-free DMEM and treated with 40 µM CI (0.2% DMSO vehicle) for 1 h under OxR conditions. For α-tocopherol (vitamin E) studies, a stock solution of vitamin E was prepared in DMSO and stored at −20°C. Cells were washed twice with serum-free DMEM and treated with 50 µM vitamin E (0.01% DMSO vehicle) for 30 min in OxR conditions. Finally, for cyclosporin A (CsA) studies, cells were pretreated with 2.5 µM CsA (0.01% DMSO vehicle) for 2 h in OxR conditions. After pretreatment with inhibitor, cells were treated with EL and transferred to hypoxic glove boxes for 24 h.

**Protein isolation.** Hepa1c1c7 and HepaC4 cells were plated in six-well tissue culture plates until 80–90% confluent. They were washed twice with serum-free DMEM and treated with EL in OxR or Hx serum-free DMEM. At various times after treatment, the serum protease inhibitor PMSF was added to the medium at a final concentration of 1 µM to neutralize EL activity. Cells were then scraped and collected via centrifugation (2,000 rpm for 2 min). The pellet was washed with ice-cold PBS. Whole cell extracts were prepared by sonication (two 5-s pulses) of pellets in ice-cold Harlow buffer containing HALT protease and phosphatase inhibitors (Thermo Scientific, Rockford, IL). Lysates were collected by centrifugation at 10,000 g for 10 min. Cyttoplasmic and nuclear extracts were prepared by lysing cells in ice for 10 min with lysis buffer A (1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, and 0.05% IGE-PAL, pH 7.5 containing HALT protease and phosphatase inhibitors). Lysates were centrifuged (3,000 rpm for 10 min), and supernatant was collected in prechilled tubes as the cytoplasmic fraction. Nuclear pellets were resuspended with lysis buffer B (5 mM HEPES, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, and 26% glycerol [vol/vol], pH 7.9), and NaCl was added to a final concentration of 300 mM. The pellets were sonicated (two 5-s pulses) and then allowed to sit on ice for 30 min. Nuclear fraction supernatant was collected in prechilled tubes after centrifugation (2,000 g, 25 min). Protein concentration was determined by the bicinchoninic acid assay (Thermo Scientific). Protein samples from the Hx-exposed cells were collected in the Hx incubator to avoid reoxygenation of cells prior to sample collection.
Western analysis. For MAPK and BNIP3 detection, 20 μg of protein were separated on 10% SDS-polyacrylamide gel. For HIF-1α detection, 25 μg of cytoplasmic and nuclear protein were loaded onto a 10% SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were washed with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) and blocked for 1 h with 5% BSA for MAPK and BNIP3 detection or 5% milk for HIF-1α detection. Blots were probed with phosphorylated p38, phosphorylated ERK1/2, phosphorylated JNK, and BNIP3 primary antibodies (Cell Signaling Technology, Beverly, MA; 1:1,000 dilution in 5% BSA) or HIF-1α antibody (Novus Biologicals, Littleton, CO; 1:500 dilution in 5% milk) at 4°C overnight. Membranes were washed with TBST, probed with secondary goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:2,000–1:10,000 dilution in 5% BSA or milk in TBST). HRP was visualized with the HyGlo chemiluminescent HRP antibody detection reagent and exposed to HyBlot CL film (Denville Scientific, Metuchen, NJ).

Membranes were stripped using Restore Western blot stripping reagent (Thermo Scientific) and reprobed for total p38, ERK1/2, JNK, and BNIP3 with phosphorylated p38, phosphorylated ERK1/2, phosphorylated JNK, and BNIP3 primary antibodies (Cell Signaling Technology, Beverly, MA; 1:1,000 dilution in 5% BSA), or lamin (Abcam; 1:10,000 dilution in 5% BSA). HIF-1α Western blots were stripped and reprobed for tubulin (cytoplasmic, 1:1,000 dilution in 5% BSA) or lamin (nuclear, 1:10,000 dilution in 5% BSA) to serve as loading controls and to determine the purity of each fraction.

RNA isolation and RT-PCR. Cells were treated with various concentrations of EL in OxR or Hx for various times. After treatment, EL was neutralized with 1 μM PMSF, and cells were collected by centrifugation. Total RNA was isolated with TRIZol reagent (Invitrogen) according to the manufacturer’s directions. RNA quantity and quality were assessed by Nanodrop 2000 (Thermo Scientific). cDNA was prepared from 1 μg of RNA with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). The expression level of specific genes was analyzed using SYBR Green (Applied Biosystems, Foster City, CA). Copy number was determined by comparison with standard curves of the respective genes. Expression level was normalized to the hypoxanthine guanine phosphoribosyl transferase (HPRT) gene. PCR primers were used as follows: mouse BNIP3 [5′-gctascaaagtctaatagcgtc-3′ (forward) and 5′-aggagtcaaagagacgctctt-3′ (reverse)], mouse Nix [5′-tgctgcgcttcagttcagaa-3′ (forward) and 5′-agggacgtcggtagctctc-3′ (reverse)], and HPRT [5′-aggagtcttgttagttgcagct-3′ (forward) and 5′-ggggacgaatagctattc-3′ (reverse)].

DNA was collected from Hx-exposed cells in the Hx incubator to avoid reoxygenation of the cells.

Malondialdehyde measurement. Hepa1c1c7 cells were treated with 0 or 7.5 U/ml EL in OxR or Hx for 16 h. After treatment, cells were collected by centrifugation and sonicated (two 5-s pulses) in cold 50 mM potassium phosphate buffer (pH 7.4, 1 mM EDTA). Samples were collected from Hx-exposed cells in the Hx incubator to prevent reoxygenation of the cells. Thiobarbituric acid reactive substances were detected in cell lysates with a kit (Cayman Chemical, Ann Arbor, MI) using malondialdehyde (MDA) for the standard curve. Results are expressed as MDA equivalents. In some experiments, cells were pretreated for 2 h with 10 μM SB-203580 or for 30 min with 50 μM vitamin E. MDA concentration was also assessed in HepaC4 cells.

Statistical analyses. Values are means ± SE. Results were analyzed using two- and three-way ANOVA as appropriate and Tukey’s post hoc test to compare groups. P < 0.05 was the criterion for significance.

RESULTS

Cytotoxicity of EL and Hx in primary HPCs and Hepa1c1c7 cells. Primary HPCs were not sensitive to EL alone at concentrations up to 17 U/ml. Similar to previously published results (20), 5% O2 was modestly cytotoxic to HPCs, and it significantly enhanced the cytotoxicity of EL at 8.5 and 17 U/ml (Fig. 1A) within 8 h of treatment. There was a statistically significant interaction between Hx and EL treatments, indicative of a synergistic response. This time is consistent with the development of liver injury in our LPS/drug models in vivo (19).

In Hepa1c1c7 cells, >7.5 U/ml EL caused cytotoxicity at 24 h, and Hx (2% O2) alone was modestly cytotoxic (Fig. 1B). Cotreatment of Hepa1c1c7 cells with Hx and EL resulted in a significant increase in cell death over either treatment alone, and there was a significant interaction between 2% O2 and >7.5 U/ml EL (Fig. 1B). A selective, irreversible inhibitor of EL (EL inhibitor III, peptide sequence N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone, 25 μM) completely abolished the cytotoxic effect of EL (data not shown), indicating that the proteolytic activity of the enzyme is required for the cytotoxicity. Neither Hx or EL alone nor Hx-EL cotreatment caused significant cytotoxicity at 6, 12, or 18 h, indicating that cytotoxicity developed rapidly between
18 and 24 h of treatment in Hx-EL-cotreated cells (data not shown).

EL caused concentration-dependent caspase-3/7 activation 6 h after treatment that was not affected by Hx (Fig. 2A), which is consistent with the time course of EL-induced caspase activation reported by others (9). Pretreatment with a nonpeptidyl caspase-3/7 inhibitor had no effect on the cytotoxicity of EL; however, it did attenuate cytotoxicity caused by Hx alone. Caspase inhibition did not protect cells from the cytotoxic interaction (Fig. 2B). Furthermore, pretreatment with 2.5 μM CsA to inhibit mitochondrial permeability transition did not prevent cytotoxicity (Fig. 2C).

**MAPK phosphorylation.** Phosphorylation of ERK, JNK, and p38 was assessed 30 min after treatment. Phosphorylation of ERK was modestly increased by Hx. Interestingly, EL decreased phosphorylated ERK irrespective of cotreatment with Hx (Fig. 3A). Phosphorylation of ERK was abolished by

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**Fig. 2. Caspase-3/7 and mitochondrial permeability transition do not contribute to cell death caused by Hx-EL cotreatment.** A: Hepa1c1c7 cells were treated with 0, 1.875, 3.75, 7.5, or 15 U/ml EL in OxR or Hx for 6 h, and caspase-3/7 activity was assessed. Values are means ± SE of 3 separate experiments performed in triplicate. B: Hepa1c1c7 cells were pretreated with caspase inhibitor (CI, 40 μM) and then treated with 0–15 U/ml EL in OxR or Hx for 24 h, and cytotoxicity was assessed. Values are means ± SE of 4 separate experiments in triplicate. C: cells were pretreated with vehicle [VEH (0.01% DMSO)] or cyclosporin A (2.5 μM) and treated with 0 or 7.5 U/ml EL in OxR or Hx for 24 h, and cytotoxicity was assessed. Values are means ± SE of 3 experiments in triplicate. *P < 0.05 vs. 0 U/ml EL in OxR. †P < 0.05 vs. 0 U/ml EL in Hx. ‡P < 0.05 vs. VEH in OxR. #Significant interaction between EL and Hx.

**Fig. 3.** Hx-EL cotreatment causes an additive increase in activation of p38, but not JNK or ERK. A: cells were treated with VEH (PBS) or 7.5 U/ml EL for 30 min in OxR or Hx, and phosphorylated (p-) and total (t-) ERK1/2 (44 and 42 kDa), JNK (42 kDa), and p38 (38 kDa) were determined by Western blot analysis. Some cells were pretreated with U-0126 (5 μM), SP-600125 (5 μM), or SB-203580 (10 μM) to inhibit ERK, JNK, or p38, respectively. B: densitometry was performed on phosphorylated and total p38 bands. Values are means ± SE of 6 separate experiments. *P < 0.05 vs. VEH in OxR. †P < 0.05 vs. VEH in Hx.
U-0126, which inhibits the upstream MAPK kinases MEK1 and MEK2, specific for ERK phosphorylation (Fig. 3A). JNK phosphorylation was not consistently affected by EL or Hx, and it was inhibited with 5 μM SP-600125, which prevents JNK-dependent phosphorylation of downstream mediators such as c-Jun (Fig. 3A). EL and Hx each increased phosphorylated p38, and there was an additive increase in phosphorylated p38 caused by EL-Hx cotreatment (Fig. 3A). Densitometry indicated that EL and Hx increased the ratio of phosphorylated to total p38 from 1 to 1.7 ± 0.4 and 2.0 ± 0.3, respectively, and EL-Hx cotreatment increased the ratio to 3.0 ± 0.76 (Fig. 3B). p38 phosphorylation was reduced by 10 μM SB-203580, which targets p38 and its downstream substrates.

Inhibition of ERK with U-0126 increased the cytotoxicity of EL alone but had no significant effect on the interaction of Hx and EL (Fig. 4A). JNK inhibition by SP-600125 had no effect on cell death in any treatment group (Fig. 4B). Inhibition of p38 with SB-203580 had no effect on cytotoxicity caused by Hx or EL, but it significantly attenuated cytotoxicity in Hx-EL-cotreated cells to the level in cells treated with EL or Hx alone (Fig. 4C).

**HIF-1α accumulation and activation.** HIF-1α protein was measured in cytosolic and nuclear fractions 30 and 60 min after treatment. EL did not alter HIF-1α protein levels in either fraction. Hx increased HIF-1α protein in the cytosolic and nuclear fractions, and cotreatment with EL further enhanced HIF-1α accumulation in both fractions (Fig. 5A). Blots were stripped and reprobed for tubulin and lamin to determine the purity of the cytoplasmic and nuclear fractions, respectively. HIF-1α and lamin band intensity were quantified in nuclear fractions. At 60 min, Hx significantly increased the ratio of HIF-1α to lamin by sixfold compared with untreated cells. Hx-EL cotreatment further enhanced the HIF-1α-to-lamin ratio by ~14-fold over control, and there was a significant interaction between EL and Hx treatments (Fig. 5B).

BNIP3 and Nix mRNAs were assessed 8 h after treatment, a time that corresponds to Hx-induced BNIP3 mRNA expression in primary mouse HPCs (22). EL did not affect expression of Nix (Fig. 6A); however, it did increase BNIP3 mRNA expression (Fig. 6B). Hx increased BNIP3 and Nix mRNA expression, an effect that was enhanced by cotreatment with EL (Fig. 6). Inhibition of p38 signaling had no effect on the increases in BNIP3 mRNA expression caused by Hx or EL alone, but it did reduce the increased BNIP3 expression after Hx-EL cotreatment (Fig. 6B).

BNIP3 protein levels were monitored 12 h after treatment. EL caused a modest increase in BNIP3 protein. Hx caused a more pronounced increase in BNIP3, an effect that was enhanced by cotreatment with EL (Fig. 7A). The intensity of BNIP3 protein was assessed by densitometry and expressed relative to lamin (loading control). EL significantly increased the BNIP3-to-lamin ratio from 1.0 to 3.1 ± 0.9. Hx increased the BNIP3-to-lamin ratio to 8.7 ± 3.5, and cotreatment with EL enhanced it further to 16.5 ± 6.6 (Fig. 8B). Inhibition of p38 phosphorylation attenuated the increase in BNIP3 protein due to Hx, and there was even greater reduction of BNIP3 protein in Hx-EL-cotreated cells (Fig. 7C).

**HIF-1α signaling.** To determine the role of HIF-1α in the cytotoxicity of Hx and/or EL, HepaC4 cells were used. HepaC4 cells were derived from Hepa1c1c7 cells and are deficient in HIF-1β/ARNT, the heterodimeric partner of HIF-1α required for competency as a transcription factor. As in Hepa1c1c7 cells, EL caused significant cytotoxicity in HepaC4 cells. However, Hx alone was not cytotoxic in HepaC4 cells, and there was no interaction between Hx and EL (Fig. 8).

BNIP3 mRNA and protein expression were also assessed in HepaC4 cells. In contrast to Hepa1c1c7 cells (Fig. 6B), EL did not alter the expression of BNIP3 mRNA in HepaC4 cells (Fig. 9A).
Hx enhanced the BNIP3-to-HPRT ratio from 0.41/0.12 in untreated cells to 1.54/0.78; however, this difference was not statistically significant. The BNIP3-to-HPRT ratio in Hx-EL-cotreated HepaC4 cells was 1.99/0.57, which was not different from that in cells exposed to OxR or Hx alone. Inhibition of p38 had no effect on BNIP3 mRNA expression by any treatment (Fig. 9A). The Hx-mediated increase in BNIP3 mRNA expression by any treatment (Fig. 9A). The Hx-mediated increase in BNIP3 mRNA was significantly less in HepaC4 than Hepa1c1c7 cells (1.54/0.78 vs. 2.91/0.28; Figs. 6B and 9A). Despite the modest increase in BNIP3 mRNA, no increases in BNIP3 protein caused by any treatment were detected after 12 h in HepaC4 cells (Fig. 9B).

Lipid peroxidation. The lipid-soluble antioxidant vitamin E protected Hepa1c1c7 cells from cytotoxicity of Hx or EL (Fig. 10A), whereas water-soluble antioxidants such as 4-OH-TEMPO, N-acetylcysteine, and desferrioxamine were not protective (data not shown). To determine if oxidative stress occurred, H2O2 was measured with the dichlorodihydrofluorescein diacetate assay. In our system, there were no detectable increases in H2O2 production due to any treatment (data not shown). Vitamin E pretreatment had no effect on p38 phosphorylation (Fig. 10B).

Lipid peroxidation was assessed by measurement of cellular MDA concentration. EL increased MDA compared with any treatment alone; however, this increase was significantly less than in cells cotreated with Hx and EL without added vitamin E (Table 1). Inhibition of p38 signaling by itself increased MDA concentration in OxR, but the increase due to EL in control cells did not occur in cells treated with SB-203580. It also reduced lipid peroxidation in Hx-EL-cotreated cells compared with controls (Table 1).

Lipid peroxidation was also assessed in HepaC4 cells. Within these cells, there were no treatment-related changes in MDA concentration. Basal MDA concentration was slightly greater in HepaC4 and Hx-treated HepaC4 cells than in Hepa1c1c7 cells. There was no significant difference in MDA concentration between HepaC4 and Hepa1c1c7 cells due to Hx-EL cotreatment (Table 1).

DISCUSSION

Hx and PMNs are important mediators of hepatocyte death in many models of inflammatory liver injury, and we demonstrated previously that Hx sensitizes primary rat HPCs to the cytotoxicity of PMN-derived EL (20), a finding that we confirmed in this study (Fig. 1A). To characterize the mechanism of this interaction in more detail, we extended this observation to Hepa1c1c7 cells. The response to Hx has been well charac-

Fig. 5. Hx/EL increases nuclear accumulation of hypoxia-inducible factor (HIF)-1α compared with Hx alone. A: cells were treated with VEH or 7.5 U/ml EL in OxR or Hx for 60 min, and cytoplasmic (Cyto) and nuclear (Nuc) protein extracts were probed for HIF-1α (105 kDa), lamin (70 kDa), or tubulin (50 kDa) protein via Western blot. B: intensity of HIF-1α and lamin were measured with ImageJ software. Values are means ± SE of 4 separate experiments. *P < 0.05 vs. VEH in OxR. #Significant interaction between Hx and EL.

Fig. 6. Hx/EL increases mRNA expression of HIF-1α-regulated genes, Nix and Bcl-2/adenovirus E1B-interacting protein 3 (BNIP3). Cells were treated with VEH or 7.5 U/ml EL in OxR or Hx for 8 h, and RNA was isolated. A: detection of Nix. B: detection of BNIP3. In some experiments, cells were pretreated with 10 μM SB-203580 to inhibit p38 activity. HPRT, hypoxanthine guanine phosphoribosyl transferase. *P < 0.05 vs. VEH in OxR. #P < 0.05 vs. VEH in Hx. %P < 0.05 vs. EL in OxR. #Significant interaction between Hx and EL.
terized in Hepa1c1c7 cells and in the HIF-1β/ARNT-deficient variant HepaC4 (3, 28). Similar to primary HPCs, Hepa1c1c7 cells were also sensitive to EL alone. Exposure to Hx (2% O2) significantly enhanced cell death caused by EL at concentrations as low as 3.75 U/ml (Fig. 1B), and there was a cytotoxic interaction between these treatments. Furthermore, EL inhibition protected Hepa1c1c7 cells from cytotoxicity, indicating that the proteolytic activity of the enzyme is required.

EL causes caspase-dependent apoptosis of lung epithelial cells (9, 36). Hx is also associated with caspase activation in cardiac fibroblasts (4). Therefore, we examined the role of intrinsic apoptosis and the effector caspases-3 and -7 in the mechanism of Hx/EL-induced cytotoxicity. EL caused a concentration-dependent increase in caspase-3/7 activation that was not enhanced by Hx (Fig. 2A); however, caspase inhibition did not protect Hepa1c1c7 cells from the cytotoxic interaction of Hx with EL (Fig. 2B). CsA also did not afford protection (Fig. 2C). These results suggest that neither caspase signaling nor mitochondrial permeability transition contributes to Hx/EL-induced cell death; thus the mode of cell death does not appear to be apoptotic.

The role of the MAPKs in cell death is complex and depends on cell type. Hx and EL activate MAPKs in several cell types,

Fig. 7. Hx/EL increases expression of BNIP3 protein in a p38-dependent manner. A: Hepa1c1c7 cells were treated with VEH or 7.5 U/ml EL in OxR or Hx for 12 h, and BNIP3 (25 kDa) and lamin (70 kDa) protein were detected via Western blot. B: BNIP3 and lamin intensity were measured with densitometry using ImageJ software. Values are means ± SE of 8 separate experiments. C: cells were pretreated for 2 h with DMSO (0.01% final concentration) or 10 μM SB-203580 to inhibit p38 and then treated with VEH or 7.5 U/ml EL in OxR or Hx for 12 h. BNIP3 protein was detected via Western blot.

Fig. 8. HIF-1β-deficient HepaC4 cells are protected from the cytotoxicity of Hx/EL. Hepa1c1c7 and HepaC4 cells were treated with VEH or 7.5 U/ml EL in OxR or Hx for 24 h, and cytotoxicity was assessed. Values are means ± SE of 4 experiments in triplicate. *P < 0.05 vs. VEH in OxR. **P < 0.05 vs. EL in OxR. #Significant interaction between treatments.

Fig. 9. HepaC4 cells do not express BNIP3 mRNA or protein after Hx-EL cotreatment. A: HepaC4 cells were pretreated with VEH (0.01% DMSO) or 10 μM SB-203580 and then treated with VEH (PBS) or 7.5 U/ml EL in OxR or Hx for 8 h. Expression of BNIP3 and HPRT mRNA was assessed by quantitative RT-PCR. Values are means ± SE of 4 experiments in duplicate. B: Hepa1c1c7 and HepaC4 cells were treated with VEH or EL in OxR or Hx for 12 h, and whole cell lysates were probed for BNIP3 protein and lamin.
and the result of activation is often cytotoxicity and/or activation of HIF-1α or other transcription factors (23). ERK is generally activated by growth factors and mitogens and is involved in cell growth, differentiation, and survival pathways, whereas JNK and p38 are activated by inflammatory cytokines and various cell stresses and are involved in inflammation, apoptosis, and oncotic cell death (29). In studies presented here, there were no consistent changes in phosphorylation of JNK by any treatment (Fig. 4A). Furthermore, JNK inhibition had no effect on the cytotoxicity of any treatment, suggesting that JNK does not play a role in the cytotoxic interaction of Hx and EL.

Interestingly, there was a high basal level of phosphorylated ERK that was reduced by EL. Hx increased phosphorylated ERK, but this was also reduced by cotreatment with EL (Fig. 3A). This is consistent with a report in which EL reduced phosphorylated ERK in endothelial cells (27). Inhibition of ERK enhanced cell death from EL alone (Fig. 4B). Depending on the concentration-response relationship for phosphorylated p38-mediated events, it is possible that this additive increase in phosphorylated p38 is sufficient to alter downstream signaling to enhance cytotoxicity in cells cotreated with Hx and EL. Inhibition of p38 attenuated the cytotoxicity of Hx-EL cotreatment, although it had no effect on the modest cytotoxicity of Hx or EL alone (Fig. 4C). This suggests that p38 is important to the signaling in Hx-EL-cotreated cells that leads to cell death and that this role is unique compared with the mechanism of either insult alone.

One mechanism by which p38 might be involved in the interaction of Hx and EL is through its ability to modulate the transactivation of HIF-1α. p38 can directly phosphorylate HIF-1α to stabilize its interaction with HIF-1β or the interaction with transcriptional coactivators (23). Consistent with this hypothesis, p38 was necessary not only for cytotoxicity in Hx-EL-cotreated cells but also for the increase in BNIP3 mRNA (Fig. 6B) and protein (Fig. 7C) under these conditions.

HepaC4 cells are derived from Hepa1c1c7 cells and are deficient in HIF-1α/ARNT; thus they are deficient in HIF-1α signaling and transactivation. HIF-1β expression is required for the formation of the HIF-1 DNA binding complex (28), as well as for HIF-1α-mediated transcription (3), in hepatoma cells. In HepaC4 cells, EL alone caused modest cytotoxicity (Fig. 8) to a level similar to that in Hepa1c1c7 cells (Fig. 1A), indicating that the cytotoxicity of EL is not mediated through HIF-1α signaling. Interestingly, Hx alone did not cause cytotoxicity in HepaC4 cells, and there was no increase in cell death upon Hx-EL cotreatment. These data suggest that HIF signaling is required for the cytotoxic interaction of Hx and EL. Furthermore, despite a modest increase in BNIP3 mRNA expression in Hx-treated and Hx-EL-cotreated HepaC4 cells (Fig. 9), there was no detectable BNIP3 protein. These data verify that HIF-1α signaling is impaired in HepaC4 cells. They also suggest that BNIP3 might have a causative role in the Hx/EL cytotoxicity. The mechanism by which BNIP3 and Nix cause cell death depends on cell type and experimental conditions. For example, in primary mouse HPCs, Hx-induced BNIP3 expression contributes to a mode of cell death that resembles oncotic necrosis and is independent of caspase activation and mitochondrial permeability transition (22).

Table 1. Effect of Hx/EL on lipid peroxidation in Hepa1c1c7 and HepaC4 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hepa1c1c7 cells</th>
<th>HepaC4 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Vitamin E</td>
</tr>
<tr>
<td>OxR + VEH</td>
<td>0.60 ± 0.22</td>
<td>0.72 ± 0.41</td>
</tr>
<tr>
<td>OxR + EL</td>
<td>1.22 ± 0.28</td>
<td>1.05 ± 0.54</td>
</tr>
<tr>
<td>Hx + VEH</td>
<td>0.72 ± 0.22</td>
<td>0.37 ± 0.06</td>
</tr>
<tr>
<td>Hx + EL</td>
<td>3.40 ± 0.62*</td>
<td>1.40 ± 0.33*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 3–5 experiments. Hepa1c1c7 cells were treated with vehicle (VEH (PBS)) or 7.5 U/ml elastase (EL) in an O2-replete (OxR) or a hypoxic (Hx) environment for 16 h and assessed for thiobarbituric acid-reactive substances [malondialdehyde (MDA) equivalents]. In some experiments, cells were pretreated with 50 μM vitamin E for 30 min or 10 μM SB-203580 for 2 h. MDA concentration was also assessed in HepaC4 cells.

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is consistent with data presented in the present study, as there was no evidence for a role of caspases or mitochondrial permeability transition (Fig. 2). Furthermore, p38 signaling was required for Hx-mediated BNIP3 expression and cell death of primary HPCs (22), which is also consistent with our results.

In Hepa1c1c7 cells, Hx-EL cotreatment enhanced lipid peroxidation compared with EL treatment alone, and vitamin E reduced the lipid peroxidation (Table 1). Furthermore, vitamin E protected against the cytotoxicity from Hx/EL exposure (Fig. 10A), suggesting that lipid peroxidation plays a role in the interaction. Lipid peroxidation is an important mechanism of HPC death due to ischemia-reperfusion (24) and sepsis, conditions in which Hx and EL are involved. Pharmacological inhibition of EL attenuated lipid peroxidation and liver injury in mouse (24) and rat (25) models of ischemia-reperfusion.

There was no measurable increase in ROS production (dichlorodihydrofluorescin fluorescence) due to any treatment in this system (data not shown). This is in contrast with previous studies in which Hx increased ROS production from HepG2 and Hep3B hepatocellular carcinoma cells (2). EL is associated with ROS production in A549 lung epithelial cells (8). One potential reason for the lack of increase in ROS production is the inherently higher level of ROS in hepatomas than normal HPCs (34). This raises a question about the source of radicals for initiation of lipid peroxidation in this model, which is not known. Others demonstrated that Hx-induced ROS can activate p38 in various cell types (5, 22), but this does not appear to be the case in Hepa1c1c7 cells in this study, since no ROS were detected. Since vitamin E did not attenuate p38 activation (Fig. 10B), radicals involved in lipid peroxidation do not appear to contribute to p38 activation. However, inhibition of p38 signaling attenuated the Hx/EL-mediated increase in MDA concentration (Table 1), suggesting that p38 might contribute to cell death through lipid peroxidation, in addition to its role in enhancing HIF-1α-mediated gene transcription.

Basal MDA concentration was greater in HepaC4 than Hepa1c1c7 cells, but this apparent difference was not statistically significant. MDA concentration was significantly greater in Hx-treated HepaC4 than Hepa1c1c7 cells; however, there was no difference in MDA concentration caused by Hx-EL cotreatment between Hepa1c1c7 and HepaC4 cells (Table 1). This indicates that Hx enhancement of EL-induced lipid peroxidation is not mediated through HIF-1α signaling.

Exposure of the HepG2 cell line to 1% O2 increased the expression of α1-anti-trypsin, among other acute-phase proteins (40), suggesting that hypoxia might also upregulate hepatic α1-anti-trypsin expression in vivo. However, we found a decrease in α1-anti-trypsin expression in livers of rats treated with LPS and sulindac, a model in which Hx has been shown to contribute to liver injury (43). This would tend to promote EL activity in the liver in that model. Furthermore, this is consistent with previously published reports that EL inhibition protects animals from LPS/drug-induced liver injury (7, 33).

On the basis of our results, a working hypothesis for the mechanism by which Hx and EL interact to cause HPC death is presented in Fig. 11. Hx-EL cotreatment results in early activation of p38 and accumulation of HIF-1α protein. p38 contributes to the transactivation of HIF-1α and, thereby, to the expression of cell death factors such as BNIP3 in Hx-EL-cotreated cells. EL-mediated lipid peroxidation also contributes to cell death, and Hx enhances this response by a mechanism that depends, at least in part, on p38. These data offer insight into the mechanism by which Hx and EL released by activated PMNs synergize in contributing to cell death. Understanding the intracellular signaling that results in HPC death is crucial to developing treatments to prevent liver failure in ischemia-reperfusion-, sepsis-, and drug-induced liver injury.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

E.M.S., P.E.G., and R.A.R. are responsible for conception and design of the research; E.M.S. performed the experiments; E.M.S. analyzed the data; E.M.S., P.E.G., and R.A.R. interpreted the results of the experiments; E.M.S. prepared the figures; E.M.S. drafted the manuscript; E.M.S., P.E.G., and R.A.R. edited and revised the manuscript; E.M.S., P.E.G., and R.A.R. approved the final version of the manuscript.

REFERENCES

Hypoxia-elastase interaction


