Human esophageal microvascular endothelial cells respond to acidic pH stress by PI3K/AKT and p38 MAPK-regulated induction of Hsp70 and Hsp27

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GASTROESOPHAGEAL REFLUX DISEASE (GERD) is the most common malady of the esophagus and ~7–10% of the US population suffers from varying degrees of this condition (44). To date, there has been extremely limited investigation into the cellular and molecular mechanisms which underlie human esophageal inflammation, as most research has focused on clinical trials in patients and whole animal studies (51). Studies focusing on cellular and molecular mechanisms in GERD have emphasized the esophageal epithelium and its response to acid injury. However, histological assessment of GERD demonstrates classic inflammatory mechanisms, including selective leukocyte recruitment and hemorrhage, suggesting a prominent role for the esophageal microvasculature in disease pathophysiology.

Inflammation is a complex biological process involving a highly orchestrated interaction among several cells of different origin and function. It is now established that “nonimmune cells,” including epithelial, mesenchymal, and endothelial cells, are not simply passive bystanders, but, like leukocytes, have a critical role in initiating and regulating inflammation. Central among these nonimmune cells, endothelial cells lining blood vessels play a critical function in the inflammatory process by regulating leukocyte recruitment and trafficking from the circulation (56). Endothelial activation in response to cytokines and bacterial products results in enhanced cell adhesion molecule expression and chemokine production, which mediate increased binding and transmigration of leukocytes across the vascular wall. Thus, the endothelium is strategically situated as an anatomic barrier in the esophageal mucosa, separating circulating immune cells from tissue interstitium. The role of esophageal endothelial activation in GERD, particularly in response to acid activation, has not been defined.

The heat shock response is an evolutionarily conserved mechanism for the maintenance of cellular homeostasis following sublethal noxious stimuli, including thermal, oxyradical, and inflammatory stress. Heat shock proteins (Hsps) are appreciated to chaperone intracellular proteins, which might otherwise be denatured during periods of chronic stress. Recent evidence has demonstrated that Hsps will chaperone signal-transduction proteins and modulate signaling cascades during periods of repeated stress (54). The ability of acidic stress to induce the heat shock response, particularly in the context of GERD, has undergone limited evaluation. We (47) have recently demonstrated that esophageal endothelial cells exposed to acidic exudate may contribute to GERD in the setting of a disturbed mucosal squamous epithelial barrier (i.e., erosive esophagitis, peptic ulceration).

esophagus; esophagitis; gastroesophageal reflux disease; microvasculature; phosphatidylinositol 3-kinase/Akt; VCAM-1

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First published June 21, 2006; doi:10.1152/ajpcell.00474.2005.—The heat shock response maintains cellular homeostasis following sublethal injury. Heat shock proteins (Hsps) are induced by thermal, oxyradical, and inflammatory stress, and they chaperone denatured intracellular proteins. Hsps also chaperone signal transduction proteins, modulating signaling cascades during repeated stress. Gastroesophageal reflux disease (GERD) affects 7% of the US population, and it is linked to preexisting Hsps in the cytoplasm. However, in stress-activated cells, such as cells exposed to acidic pH stress by PI3K/AKT and p38 MAPK-regulated induction of Hsp70 and Hsp27. © 2006 the American Physiological Society
to elevated temperatures, the Hsps are dissociated from HSF1, forming homotrimeric HSF1 complexes that, in the proper phosphorylation state, bind consensus sites on DNA and activate transcription (6). The mechanisms controlling HSF1 activation are not well understood, but HSF1 activity is modulated by phosphorylation (20). HSF1 activity can be negatively regulated by Ser203 and Ser307 phosphorylation (30, 31). Mutation of Ser307 to alanine in HeLa cells resulted in constitutive activation of HSF1 (30, 31). In NIH 3T3 cells and human monocytes, phosphorylation of HSF1 on Ser303 by glycogen synthase kinase-3β (GSK-3β) leads to phosphorylation of Ser307 by the ERKs (10). In HeLa cells, overexpression of GSK-3β resulted in HSF1 inactivation (22). Thus, in some cell types, GSK-3β may be a negative regulator of HSF1 activity and the subsequent expression of Hsps. GSK-3β can be inhibited by activation of Wnt and phosphatidylinositol 3-kinase (PI3K) pathways (11, 12). Activated PI3K can phosphorylate and activate Akt (1). In turn, activated Akt can phosphorylate Ser9 of GSK-3β, which may inhibit its own activity (14). Akt also can be activated by other stressors, such as oxidative stress, osmotic stress, and heat shock, suggesting negative feedback involving various mechanisms of activation (32, 52). Thus the complex contribution of PI3K activity to heat shock-induced Akt activation appears seemingly contradictory and has resulted in ambiguity about the specific signals that regulate stress-induced Akt activation, and the subsequent regulation of the downstream HSF1.

In the present study, we used primary cultures of HEMEC (47) to characterize the role of MAPK signaling pathways and Hsps in esophageal endothelial activation following prolonged exposure to acidic pH. We demonstrate induction of Hsp27 and Hsp70 at the level of mRNA and protein in HEMEC exposed to acidic pH, which was blocked by the p38 MAPK inhibitor SB-03580. Functionally, acidic pH exposure resulted in inflammatory activation of HEMEC as evidenced by VCAM-1 expression. Hsp expression in response to acidic pH also induced cell survival mechanisms in HEMEC, downregulating apoptosis in response to serum withdrawal. Here we have demonstrated acidic pH induced activation of Akt through PI3K activity in HEMEC. Moreover, HSF1 activation was also dependent on PI3K activity, leading to increased expression of Hsp70. Our data suggest that acidic exposure, in addition to effects on the esophageal epithelium (55), will also play a role in the induction of the heat shock response and endothelial activation in the human esophagus and may contribute to pathophysiological mechanisms in GERD.

MATERIALS AND METHODS

Patients. Normal esophageal full-thickness specimens were obtained from discarded cadaverous tissue from transplant donors as well as normal margins from patients that had undergone esophagectomies. The use of human tissues for isolation of HEMEC was approved by the Institutional Review Board of The Medical College of Wisconsin.

Reagents. Endothelial cell growth supplement was from UpState (Charlottesville, VA). RPMI 1640 medium and fetal bovine serum were obtained from BioWhittaker (Walkersville, MD). Human plasma fibronectin was purchased from Chemicon International (Temecula, CA). MCDB-131 medium, porcine heparin, and penicillin/streptomycin/fungizone were purchased from Sigma (St. Louis, MO). Unless otherwise indicated, all other chemicals used in this study were purchased from Sigma.

Cell culture. HEMEC were isolated using a previously described technique (7, 47). HEMEC cultures were recognized by microscopic morphologic features, expression of factor VIII associated antigen and modified lipoprotein uptake using 1,1-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate-labeled LDL (Biomedical Technology, Stoughton, MA) and fluorescence microscopy (58). All experiments were carried out using primary endothelial cell cultures between passages 8 and 14. Cell viability and purity was ≥95%, as assessed by trypan blue exclusion. Similar protocol was used for human intestinal microvascular cell (HIMEC) isolation (7, 47).

RNA extraction and semi-quantitative RT-PCR. Hsp27, Hsp70, and constitutive heat shock 70 (Hsc70) gene expression were assessed in unstimulated control and activated confluent cultures of HEMEC, with or without pharmacological inhibitors. Endothelial cells were exposed to 42°C for 30 min or to acid for 60 min, and then they were incubated in media (pH 7.2) for specified time points at 37°C. Total RNA was extracted using TriZOL LS (Invitrogen, Carlsbad, CA) and quantitated by optical density. One microgram of total RNA was reverse transcribed using SuperScript II RT (GIBCO-BRL), Grand Island, NY) in a total reaction volume of 20 μl. One microliter of reverse-transcription product (cDNA) was PCR amplified using Ampli-Taq DNA polymerase (Perkin Elmer, Norwalk, CT) and 0.5 μl each of 10 μM Hsp27, Hsp70, and Hsc70 forward and reverse primers. β-Actin primers were included in the reaction mixtures as an internal control for the efficiency of the RT and the amount of RNA used in the RT-PCR. PCR cycle consisted of a denaturation step (94°C, 1 min), an annealing step (60°C, 1 min), and an elongation step (72°C, 1.5 min) with a total of 35 cycles, followed by an additional extension step (72°C, 7 min). The primer sequences are shown in Table 1.

PCR products were run on 1% agarose gels and stained with 0.5 μg/ml ethidium bromide, visualized under UV light, and then photographed.

SDS-PAGE and Western blot analysis. Gel electrophoresis and Western blot analysis was performed as described previously (46). In brief, HEMEC were analyzed either unstimulated or following activation, as specified. Confluent HEMEC monolayers were then lysed and equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with specific MAPK antibodies, p44/42 MAPK, p38 MAPK, JNK, and Akt (phosphorylated and nonphosphorylated), respectively (Cell Signaling Technology, Beverly, MA), anti-VCAM-1 (R&D Systems, Minneapolis, MN), HSF1 antibody (Chemicon), Hsps antibodies

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Length (bp)</th>
<th>Cycles</th>
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<tr>
<td>Human Hsp27</td>
<td>Forward: 5’-3’ GCT-TTG-CCG-ATG-AGT-GCT-CTC</td>
<td>176 bp</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTC-GGC-CTG-TGT-GAG-TGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human Hsp70</td>
<td>Forward: 5’-3’ CCA-TGG-TGC-TGA-AGA-TGA-AG</td>
<td>284 bp</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Reverse: CAG-CAG-CGT-CGA-GTG-AGA-CC</td>
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<tr>
<td>Human Hsc70</td>
<td>Forward: 5’-3’ CCA-TGG-TGC-TGA-AGA-TGA-AG</td>
<td>283 bp</td>
<td>25</td>
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<td>Reverse: TGG-TGC-ATC-GTC-AGG-ATG-GAC-AC</td>
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<td>Human β-actin</td>
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<tr>
<td></td>
<td>Reverse: CTT-TGG-TGG-AGG-TGT-AG</td>
<td>436 bp</td>
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Hsp, heat shock protein.
(Santa Cruz Biotechnology, Santa Cruz, CA) or phospho-Hsp27 (UpState). Immunodetection of bound primary antibody was performed by HRP-conjugated secondary antibody and ECL (Amersham Pharmacia Biotech, Arlington Heights, IL).

p38 MAPK activity assay. p38 MAPK activity was assayed in cell lysates using a nonradioactive kinase activity assay kit (Cell Signaling Technology) (45). Briefly, active p38 MAPK was immunoprecipitated from lysates with immobilized phospho-p38 MAPK (Thr180/Tyr182) monoclonal antibody. In vitro kinase assay of p38 MAPK activation was determined by measurement of its catalytic activity with the use of the in-gel kinase assay using, GST-MAPKAPK-2 Fusion Protein and recombinant Hsp27 as substrate according to the manufacturer’s instructions.

Two-dimensional gel electrophoresis. Isoelectric focusing (IEF) was performed in a Hoefer unit (GE Health Care-Amersham Pharmacia, San Francisco, CA). In brief, the cells were lysed in 8 M urea, 0.5% CHAPS, 60 mM dithiothreitol (DTT), 2% Pharmalyte (Amersham Biosciences) pH ranging from 3–10 or 4–7. Equal amounts of protein from cell lysates obtained from different treatment groups were immobilized with IPG (Amersham Biosciences) buffer. After IEF, the strips were equilibrated first with 2% (wt/vol) SDS, 50 mM Tris·HCl, pH 8.8, 6 M urea, 30% (vol/vol) glycerol, 0.002% bromophenol blue, and 10 mg/ml DTT, and then with the same buffer containing 25 mg/ml iodoacetamide instead of DTT. After equilibration, each IEF gel was overlaid on a single well 10–20% gradient IPG SDS-polyacrylamide gel (Bio-Rad, Hercules, CA), electrophoresed, and blotted as described above. The membranes were then probed either with an antibody against phosphorylated and nonphosphorylated Hsp27 or Hsp70. The immunoreactive spots were visualized using a secondary antibody conjugated to horseradish peroxidase and ECL.

Pharmacological modulation of HEMEC. The contribution of signal transduction pathways to HEMEC activation and gene expression were defined using specific inhibitors, including: p38 MAPK inhibitor SB-203580 (5 μM; Calbiochem, San Diego, CA); p44/42 MAPK inhibitor PD-98059 (10 μM; Calbiochem); JNK inhibitor SP-600125 (10 μM; Calbiochem); and the heat shock inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG; 2 μM; Calbiochem). HEMEC were either incubated in acidified growth medium (pH 4.5) by 10.220.33.6 on August 27, 2017 http://ajpcell.physiology.org/ Downloaded from for the indicated times and conditions. The cells were fixed with 3.7% (vol/vol) formaldehyde in PBS for 20 min at room temperature, permeabilized with Triton X-100 (0.1% (vol/vol) in PBS) for 10 min, blocked with 2.5% (wt/vol) BSA/PBS, and stained with fluorescein-isothiocyanate-streptavidin (Pierce Biotechnology) and fluorasein isothiocyanate-streptavidin (Pierce Biotechnology). Slides were mounted and visualized with the use of a fluorescence microscope (Olympus BX-40).

Actin filament assembly. F-actin polymerization was assessed in HEMEC as described previously (45). Briefly, serum-starved HEMECs were heat shocked at 42°C or exposed to acidic pH (pH 4.5) for 10 min or thermal stress as described above. The cells were then incubated at 37°C in low-serum media (5% fetal bovine serum) for 10 days. After the 10-day culture period, five random high-power fields in the HEMEC monolayers were counted using an ocular grid following staining with trypan blue, as previously described (23). For the cell adhesion assay, HEMEC were grown and treated as above, and then when they were washed and fixed in 1% paraformaldehyde. The percentage of apoptotic cells was evaluated using a TdT-mediated dUTP nick end labeling (TUNEL) assay kit according to manufacturer’s instruction (In Situ Cell Death Detection Kit, POD, Roche Diagnostics, Indianapolis, IN).

Assessment of cell adhesion molecule surface expression. Surface expression of VCAM-1 was performed by using radioactive immunoassay as described previously (46, 47).

HSF1 gel EMSA. HEMEC grown on 60-mm plates were stimulated with acidic pH 4.5 for 1 h and washed twice with ice-cold PBS, and nuclear protein extracts were prepared using NE-PER a nuclear protein extraction kit from Pierce Biotechnology. For the DNA binding assay, an end-labeled biotinylated double-stranded HSE-1 oligonucleotide (5′-ATA AGA AAG AAA TAT GGA ATT TTC GGA-3′) was obtained from Integrated DNA Technologies (Coralville, IA). Binding reactions were carried out using nonradioactive LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology) according to the manufacturer’s protocol. Reaction products were separated through a 6% DNA retardation gel (Invitrogen), transferred to Biodyne B membrane (Pierce). Membranes were exposed to X-ray films and were developed using Chemiluminescent Nucleic Acid Detection Module (Pierce Biotechnology).

RESULTS

Hsp27, Hsp70, and Hsc70 mRNA expression in HEMEC. We determined the patterns of heat shock protein (Hsp27, Hsp70, and Hsc70) expression in HEMEC following acid and thermal stress (i.e., 42°C heat) exposure. With the use of semiquantitative RT-PCR and their respective primers, levels of Hsp27, inducible Hsp70 and constitutive Hsc70 mRNA were examined in control and activated HEMEC. The levels of mRNA between control and activated HEMECs were compared by densitometric analysis after normalization to β-actin, which functioned as an internal control. Hsp27 mRNA expression was increased 8- to 10-fold following either acidic or thermal heat stimulation of HEMEC, but it was minimally expressed in unstimulated control HEMEC (Fig. 1A). HEMEC Hsp70 mRNA expression was robustly increased 8- to 12-fold following either acidic or thermal heat stimulation, but it was again minimally expressed in unstimulated control HEMEC (Fig. 1B). Hsc70 mRNA was expressed in both unstimulated control and stimulated HEMEC. However, no detectable differences in the level of Hsc70 mRNA expression were detected between control and activated HEMEC (not shown). The induction of both Hsp27 and Hsp70 mRNA by pH 4.5 was time
dependent and was maximized by 3 h and 12 h, respectively (Fig. 1C). Thermal stress also resulted in Hsp27 and Hsp70 mRNA expression in a similar time frame (data not shown). β-Actin mRNA expression showed comparable density of bands for both control and activated HEMEC. The amplified cDNA sizes for Hsp27, Hsp70, Hsc70, and β-Actin were 176, 284, 283, and 436 bp, respectively. These data indicate that the organ-specific esophageal microvascular endothelial cells respond to the stress of either heat or acid by increasing expression of mRNA for Hsp27 and Hsp70 but not Hsc70.

Hsp27 and Hsp70 protein expression in HEMEC. Because acidic exposure changes mRNA levels for Hsp27 and Hsp70, we next sought to determine whether this would translate into similar patterns of protein expression for these heat shock proteins. HEMEC cell lysate from unstimulated control cultures and cells exposed to pH 4.5 were prepared and analyzed for Hsp27 and for constitutive and inducible Hsp70 by SDS-PAGE and Western blot analysis. Using antibodies to human Hsp27, we demonstrated that exposure to acidic pH (pH 4.5) increased the level of Hsp27 protein in HEMEC by 3 h, which was maximized by 6 h and declined to basal levels by 24 h (Fig. 2A). We also detected changes in Hsp70 protein levels in the HEMEC exposed to acidic pH, the Hsp70 protein expression maximized 12–18 h after stimulation and slightly de-
creased by 48 h (Fig. 2B). Thermal stress had similar effects on HEMEC (data not shown). In contrast, there were no detectable differences in the level of constitutive Hsc70 protein in HEMEC when we compared unstimulated control cells, or cultures exposed to thermal (i.e., 42°C) or acid stress (not shown). Figure 2C demonstrates the differential Hsp70 protein expression in response to acidic pH between HEMEC and HIMEC, which are derived from the lower gut.

MAPK signaling in HEMEC. Next, we sought to characterize the signaling mechanisms involved in acidic pH activation of HEMEC. We have previously demonstrated that short-duration exposure of HEMEC to acid (1–5 min) would induce activation of these cells, with activation of JNK/SAPK, but not p38 MAPK (47). It has also been demonstrated that longer duration of acid exposure significantly enhances phosphorylation of MAP kinases in esophageal epithelium and isolated epithelial cells in vitro, thus implicating acid exposure with cellular activation mechanisms linked with carcinogenesis in Barrett’s esophagus (55). To determine whether the duration of acid exposure may also lead to activation of these additional signaling pathways in HEMEC, we exposed cells to pH 4.5 for multiple, longer time points with acidified growth medium. MAPK activation was assessed in HEMEC lysates using phosphorylated and nonphosphorylated antibodies to MAPK families. Although esophageal microvascular endothelial cells should not normally be exposed to acidic pH in vivo, we hypothesized that these cells located in the esophageal submucosa might become exposed to acidic pH in the setting of a disturbed mucosal squamous epithelial barrier, which is found in erosive reflux esophagitis and its associated complications, including peptic esophageal ulceration.

When HEMEC were exposed to acidic pH for various time periods, there was a rapid and significant activation of JNK which was seen as early as 1 min. Activation of p44/42 MAPK was first detected at 15 min and maximized by 30 min, while activation of p38 MAPK was delayed, appearing at 60 min (Fig. 3A). These experiments suggest that exposure of esophageal microvascular endothelial cells to acidic pH will result in transient early activation of JNK and late activation of p38 MAPK if prolonged acidic exposure is encountered in the esophagus. We confirmed that equal amounts of p38, p44/42 MAPK, and JNK proteins were analyzed by stripping and reprobing the same blots with non-phospho anti-p38, anti-p44/42 MAPK, and anti-JNK antibodies (Fig. 3A).

Western blot analysis from HEMEC which were transfected with p38 MAPK siRNA and then exposed to acidic pH, revealing loss of phosphorylated p38 MAPK expression (Fig. 3B).

p38 MAPK in vitro kinase assay in HEMEC. The activation of p38 MAPK plays a protective role during adaptation to ischemic preconditioning by phosphorylating MAPKAPK-2, which in turn phosphorylates Hsp27 (40). To determine whether this homeostatic pathway is present in HEMEC, we probed control and acidic pH stimulated HEMEC for changes in MAPKAPK-2 and Hsp27. A kinase activity assay of acid activated HEMEC immunoprecipitate demonstrated phosphorylation of the substrate Hsp27 by MAPKAPK-2 in HEMEC, which is an indirect confirmation of p38 MAPK activity (Fig. 3C). Hsp27 was not activated in control resting HEMEC.

Two-dimensional gel analysis of Hsps in acid-activated HEMEC. Hsp27 is a known substrate of p38 MAPK, and it has been shown to regulate the actin cytoskeleton. We tested the possibility that Hsp27 undergoes phosphorylation in acid-exposed HEMEC. To determine whether endogenous Hsp27 phosphorylation is increased in HEMEC stressed with acid, cell lysates from resting and acid exposed samples were analyzed by two-dimensional (2D) electrophoresis, followed by immunoblot analysis with a phospho-Hsp27 antibody. Phosphorylation causes a protein to become more acidic, thus reducing its isoelectric focusing point, and differentially phosphorylated Hsp27 (non-, mono-, bi-, or triphosphorylated) can thus be resolved by 2D gel electrophoresis (5). The need to use the isoelectric focusing stems from the fact that Hsp27 phosphorylation using phosphospecific antibodies will not differentiate phosphoepitopes found in mono-, bi- or triphosphorylated Hsp27. The different spots shown in Fig. 4A reflect nonphosphorylated (1) as well as phosphorylated (2, 3) forms of Hsp27, with the triphospho-Hsp27 being most acidic and migrating farthest to the left. As shown in Fig. 4A, the relative amount of phosphorylated Hsp27 increased significantly by 1 h in HEMEC exposed to acidic pH compared with resting cells. Further identification of Hsp70 proteins from control and acidic pH-treated HEMEC was performed by 2D gel and Western blot analysis using specific Hsp70 antibodies. Figure 4B demonstrates the increased expression of slightly basic Hsp70 in response to acidic pH.

Effect of pharmacological inhibitors and siRNA on acidic pH-induced Hsps in HEMEC. We then determined the effect of protein kinase inhibitors and gene silencing using siRNA technology on the activation of specific signaling pathways in relation to Hsp27 and Hsp70 expression in acid-treated HEMEC by SDS-PAGE and Western blot analysis. As shown above, acidic pH resulted in increased levels of Hsp27 and Hsp70 at the level of both mRNA and protein. SB-203580, a specific inhibitor of p38 MAPK (5 μM) and p38 MAPK siRNA, both blocked Hsp27 and Hsp70 mRNA and protein expression. In marked contrast, PD-098059 (10 μM) an inhibitor of MEK/p44/42 MAPK and SP-600125 (10 μM), a specific JNK inhibitor had no effect on Hsp27 mRNA and protein expression in the acid-activated HEMEC (Fig. 5A). Both PD-098059 and SP-600125 reduced Hsp70 mRNA and protein expression in the acid-activated HEMEC, but they were not as effective as SB-203580, which blocked Hsp70 mRNA and protein expression (Fig. 5B).

Both LY-294002 and Akt siRNA gene silencing blocked Hsp70 expression. The inhibitory effect of both p38 MAPK and Akt siRNA on both Hsp27 and Hsp70 protein expression are demonstrated in Fig. 5C. These data suggest that PI3K/Akt and p38 MAPK activation may play a role in esophageal inflammation and GERD in response to acidic refluxate, through mechanisms involving the activation of the microvascular endothelium.

Immunolocalization of Hsp27 and Hsp70 in HEMEC. As demonstrated in Fig. 6A, acidic pH activation of HEMEC leads to translocation of Hsp27 from the cytosol to the nucleus as detected by immunofluorescent staining using specific antibody to Hsp27 followed by a biotinylated secondary antibody and fluoroscein isothiocyanate-streptavidin. Similarly, acidic pH activation of HEMEC leads to nuclear translocation of...
Fig. 3. Role of protein kinases in HEMEC in response to acidic pH. A: Western blot analysis of HEMEC lysates using phosphorylated (p) and nonphosphorylated antibodies demonstrated that acidic pH (4.5) induced p38 MAPK phosphorylation at 60 min, which declined at 120 min. Phosphorylation of p44/42 MAPK was maximized at 30 min and declined at 60 min. JNK phosphorylation by acidic pH in HEMEC was detected as early as 1 min and declined at 5 min. Thermal stress (42°C) exerted similar effects on activation of MAPKs in HEMEC. Data are representative of 3 blots performed for each antibody. B: Western blot analysis of HEMEC lysate transfected with p38 MAPK small interfering (si)RNA shows the inhibition of phospho-p38 MAPK. C: acidic pH activates MAPKAPK-2 and phosphorylates Hsp27. In vitro kinase assay of p38 MAPK immunoprecipitate shows phosphorylation of substrate Hsp27 by MAPKAPK-2 in HEMEC. Representative results from 3 independent experiments are shown.

Fig. 4. Two-dimensional (2D) gel analysis of Hsp27 and Hsp70 in HEMEC. Samples from control unstimulated, acidic pH activated, and SB-203580 (p38 MAPK inhibitor)-pretreated HEMEC were analyzed by 2D SDS-PAGE and Western blot analysis using phospho-Hsp27 and Hsp70 antibodies. A: 2D blot demonstrates the increase in Hsp27 phosphorylation following acidic pH activation; spot 1 refers to the nonphosphorylated form, while spots 2–4 demonstrate the phosphorylated form of Hsp27, which were inhibited by SB-203580 pretreatment of HEMEC before acidic activation. B: 2D blot demonstrating the extent of Hsp70 modification in HEMEC following acidic pH and pretreatment with SB-03580. Representative results from 3 independent experiments are shown. IEF, isoelectric focusing.
Hsp70 detected by immunofluorescent staining using specific antibody to Hsp70 (Fig. 6B).

**Acidic pH alters actin cytoskeleton in HEMEC.** Hsp27 is known to interact with actin, and activation of this protein has been shown to modulate cytoskeletal organization (21). Because acidic pH resulted in increased expression of Hsp27 mRNA and protein levels in HEMEC, we began experiments investigating the effects of acidic pH on endothelial stress-fiber assembly and cytoskeletal architectural rearrangement. Using fluorescein-conjugated phalloidin, which binds to actin fibers, we assessed the distribution of actin in thermal stress and acid treated HEMEC. Our data demonstrate that exposure of HEMEC to both 42°C and pH 4.5 resulted in a shift in filamentous actin from a weblike structure in control HEMEC to a classic pattern of parallel stress fibers (Fig. 7, A and B). SB-203580, an inhibitor of p38 MAPK effectively blocked the actin filament formation in both heat-stressed (i.e., 42°C) and acid-treated HEMEC, whereas PD-098059 had no effect on fiber formation (Fig. 7, A and B). Therefore, the p38 MAPK/Hsp27 pathway plays a central role in the acidic pH stress-induced F-actin rearrangement, which may lead to increased endothelial barrier permeability.

**Prolonged acidic pH exposure results in cell adhesion molecule expression in HEMEC.** We (47) have previously demonstrated that increased cell adhesion molecule expression in response to cytokines and bacterial products results in increased leukocyte binding patterns in HEMEC. We have also demonstrated that VCAM-1 is not expressed on the surface of resting HEMEC but that it will undergo activation in response to classic inflammatory mediators. In the present studies, we investigated whether acidic pH exposure would result in increased VCAM-1 expression in HEMEC. Western blot analysis of HEMEC lysates demonstrated that acidic pH (60 min, pH 4.5, followed by 6-h incubation in routine growth media) resulted in increased VCAM-1 expression as detected by specific antibody to VCAM-1 (Fig. 8A). VEGF and TNF-α/LPS-stimulated HEMEC lysates served as negative and positive controls respectively. Densitometric analysis revealed a three-fold increase in VCAM-1 expression over the control endothelial cells (Fig. 8A). Equivalent protein loading was demonstrated using Coomassie blue staining of the gels. Next, we examined the VCAM-1 expression in HEMEC transfected with Akt and p38 MAPK siRNA. Silencing of both pathways abolished the VCAM-1 expression in HEMEC following acidic activation (Fig. 8B). Ponceau S staining of the same membrane demonstrates equivalent protein loading. Differential VCAM-1 expression in response to pH 4.5 stimulation between HEMEC and HIMEC is shown in Fig. 8C.

Fig. 5. Role of protein kinases in acidic pH-induced Hsp27 and Hsp70 in HEMEC. Protein kinase inhibitors, including 5 μM SB-203580 (p38 MAPK inhibitor), 10 μM PD-098059 (MEK/p44/42 MAPK inhibitor) and 10 μM SP-600125 (JNK/SAPK inhibitor) followed by acidic pH (4.5) exposure in HEMEC demonstrate varying degrees of inhibition on protein kinase activation. A: SB-203580 abolished Hsp27 protein in HEMEC, whereas inhibitors of p44/42 MAPK and JNK/SAPK did not exert any effect on Hsp27 protein expression. Results are representative of 3 independent experiments. B: p38 MAPK inhibitor, SB-03580 also inhibited Hsp70 protein expression in HEMEC activated by acidic pH. Inhibitors of p44/42 MAPK and JNK partially inhibited Hsp70 protein expression in HEMEC. Results are representative of 3 independent experiments. C: Western blot analysis demonstrated that neither p38 MAPK siRNA nor Akt siRNA transfected and acid-activated HEMEC expressed Hsp27 and Hsp70 protein.
Prolonged acidic pH results in HEMEC survival. We examined whether the induction of Hsps in response to acidic pH exposure would alter HEMEC physiology. Hsps are known to exert protective effects on cell survival and protection against apoptosis in multiple cell types, including endothelial cells (49). We used two measures of cell survival: enumeration of the cells in a monolayer following serum deprivation, and in situ immunohistochemical detection of DNA strand breaks (TUNEL technology) again following serum withdrawal. HEMECs that underwent prolonged exposure to acidic pH (pH 4.5) for 60 min or thermal stress for 30 min showed significantly higher survival after 10 days of incubation (Fig. 9, A and B, right). The cytoprotective effect of acidic pH was diminished by pretreatment of the cells with the nonspecific Hsp inhibitor 2 μM of 17-AAG. TUNEL assay analysis demonstrated that HEMECs exposed to acidic pH had fewer apoptotic cells (11%) compared with controls (25%) following serum deprivation. HEMEC exposed to thermal stress showed fewest TUNEL-positive cells (10%). 17-AAG treatment of HEMEC resulted in 65–75% apoptotic cells by 3 days (Fig. 9B, left).

Prolonged acidic pH exposure of HEMEC results in Akt phosphorylation. We investigated the role of the PI3K/Akt pathway in HEMEC exposed to acidic pH. Control HEMEC demonstrate a low level of phosphorylated Akt (Fig. 10A), whereas prolonged acidic pH exposure of HEMEC resulted in strong Akt phosphorylation. PI3 kinase inhibitor, LY-294002 (10 μM) pretreatment of HEMECs before acid exposure resulted in inhibition of Akt activation. Similarly, transfection of HEMEC with Akt siRNA before acid exposure resulted in silencing of Akt activation (Fig. 10B). To confirm that 17-AAG will inhibit the expression of Hsps in HEMEC, Western blot analysis was performed. The pretreatment of HEMEC with 17-AAG for 30 min before activation with acidic pH demonstrated inhibition of Hsp70 (Fig. 10C). These results suggest that acidic pH exposure protects HEMEC through the induction of Hsps and activation of the PI3K pathway.

Acid-induced activation of HSF1 in HEMEC: role of p38 MAPK and Akt. The transcription factor HSF1 is now appreciated to play a central role as a master regulator of the heat shock response and the induction of numerous Hsps in various
cell types. Initial experiments demonstrated that HSF1 was not detected in resting HEMEC but that it was readily identified in lysates of cells exposed to acidic pH for 3 h (Fig. 11A). In contrast, the transcription factor HSF2 did not undergo activation in response to acid exposure (not shown). We performed Western blot analysis to further define the signaling pathways and the expression of HSF1 in HEMEC in response to acid. In resting HEMEC, a 70-kDa protein was seen, which corresponded to unphosphorylated HSF1. Following acid pH exposure, two distinct bands were detected (i.e., 70 and 80 kDa) in HEMEC. HSF1 expression in the acid-activated HEMEC was decreased with inhibition of p38 MAPK as well as PI3K inhibition using siRNA (Fig. 11A). The detection of 70- and 80-kDa bands of HSF1 on Western blot analysis corresponds with alterations in phosphorylation state and different rates of protein mobility on SDS gel as previously described (33). The activation of HSF1 and nuclear protein DNA binding was confirmed using a nonradioactive, end-labeled biotinylated double-stranded heat shock element (HSE)-1 oligonucleotide binding assay. In these experiments, the acid activated HEMEC nuclear extract bound the oligonucleotide and this complex was detected using the Chemiluminescent Nucleic Acid Detection Module (Fig. 11B). These experiments confirm that HSF1 undergoes activation in HEMEC exposed to acid, and will bind to DNA during endothelial activation.

Next, we performed immunofluorescence staining of HEMEC monolayers following pretreatment with SB-203580 and LY-294002 and acidic exposure using anti-HSF1 antibody. In control resting cells show mainly cytosolic staining (Fig. 11C, b), whereas pH 4.5 resulted nuclear staining in HEMEC (Fig. 11C, c). Pretreatment of cells with either SB-203580 or LY-29004 diminished HSF1 nuclear staining (Fig. 11C, e and f).

DISCUSSION

In the present study, we characterized the effect of prolonged acidic pH exposure on primary human esophageal endothelial cells, defining a role for activation of MAPK family members as well as Hsps. These findings are significant, because HEMEC, the relevant microvascular endothelial cell population isolated from the human esophagus and the clinical condition GERD, appear uniquely sensitive to acid stimulation, which was not seen in lower gut-derived human microvascular endothelial cells. We focused on short- (1–5 min) and long-term (30–60 min) acid exposure, which also represents a physiological stimulus, as patients with GERD undergoing ambulatory esophageal pH monitoring will commonly demonstrate up to 1 h of acidic refluxate during sleep (13). Using this strategy for acidic pH stimulation, we demonstrated activation of both MAPK and Hsp in endothelial cells. This acidic pH activation correlated with altered cellular function in HEMEC involving inflammatory (i.e., induction of the leukocyte cell adhesion molecule VCAM-1 via MAPK activation) and cell

Fig. 7. Stress fiber assembly in HEMEC following acidic pH activation. Acidic pH (pH 4.5)-induced stress-fiber assembly was assessed by fluorescence staining with fluorescein phalloidin, a substance that specifically detects F-actin. A: confluent HEMEC monolayers were grown on fibronectin-coated glass chamber slides, and exposed to acidic pH (pH 4.5) for 1 h before staining. Control, minimal stress fibers are demonstrated in unstimulated HEMEC (negative control). Acidic pH exposure strongly increased stress-fiber assembly. At some intercellular junctions, a marked retraction of endothelial cells is notable, leading to scattered interruptions in continuity of the endothelial cell monolayer. Preincubation of HEMEC with SB-203580 (p38 MAPK inhibitor, 5 μM) markedly attenuated stress-fiber assembly by acidic pH (note diminished fluorescence intensity and less pronounced stress fiber induction). PD-098059 (p44/42 MAPK inhibitor, 10 μM) did not exert an inhibitory effect on stress-fiber assembly. B: HEMEC exposed to thermal stress also demonstrated similar patterns of stress-fiber assembly. Fluorescence microscopic images were obtained with an original magnification of ×400, at a fixed shutter speed. Representative images from 1 of 3 independent experiments are shown.

A

Control

pH 4.5

SB203580

PD098059

B

37°C

42°C

SB203580

PD098059

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survival mechanisms (i.e., prevention of apoptosis via expression of Hsp70 and activation of PI3K/Akt). Our studies demonstrate that the MAPK cascades are involved in the regulation of Hsp27 and Hsp70 in acid-activated HEMEC, which suggests a role for the microvascular endothelium in esophageal leukocyte infiltration and adaptation to the chronic inflammatory stress, which characterizes esophagitis and GERD.

To date, there has been limited investigation regarding the microvasculature and local endothelial cell populations in the pathophysiology of esophageal inflammation and GERD. Because ultrastructural studies using transmission electron microscopy (50) in patients with esophagitis showed microangiopathy in all cases assessed and GERD is associated with tissue accumulation of selective leukocyte populations (i.e., eosinophils and neutrophils), there is solid rationale for defining the role of the microvasculature and endothelial activation in these disorders (19, 37). We have carried out a systematic investigation of isolated HEMECs, demonstrating that this endothelial population is phenotypically and functionally distinct from lower gut microvascular endothelial cells (i.e., HIMEC) (47). In this initial work, short-term acid exposure of HEMEC (1–5 min) resulted in activation of JNK/SAPK, which was not seen in HIMEC cultures. In the present study, we have expanded these initial findings, focusing on mechanisms of HEMEC activation in response to prolonged acidic pH activation (up to 60 min), defining the contribution of Hsps, specifically Hsp27 and Hsp70, and the role of MAPK and PI3K/Akt pathways in endothelial activation.

Esophagitis and GERD are presently believed to result from the prolonged contact of acidic gastric refluxate with the esophageal mucosal lining, which is typically encountered nocturnally. Twenty-four-hour ambulatory pH monitoring in healthy controls and patients with GERD, which records the exposure of the esophageal lumen to gastric refluxate, has demonstrated prolonged acid retention in the esophagus during sleep (13, 15). We hypothesized that the damaged esophageal mucosal surfaces would allow for acid activation of the local esophageal microvascular populations in the esophageal wall, and we attempted to model this phenomenon in vitro using HEMEC, the relevant endothelial population.

Investigation of the cellular and molecular mechanisms which follow acid exposure have been centered on epithelial cells focusing around reparative and proliferative effects on the epithelium. Of particular interest has been the characterization of acid exposure and its relationship to metaplastic intestinal transformation of the squamous epithelium, Barrett’s esophagus, which is a precursor lesion of esophageal adenocarcinoma, the most dreaded complication of GERD. Investigation has demonstrated that acidic pH promotes cell growth in explants of Barrett’s tissue maintained in organ culture (17, 18, 43). Furthermore, Souza and colleagues (55) have characterized the activation of MAPK pathways in response to acid in SEG-1 cells, a Barrett’s adenocarcinoma-derived esophageal epithelial cell line, showing that transient exposure of these transformed epithelial cells to acidic pH resulted in cell proliferation and

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**Fig. 8.** Acidic pH-induced cell adhesion molecule expression in HEMEC. A: Western blot analysis of prolonged acidic pH activated HEMEC lysates, demonstrates increased levels of VCAM-1 protein, while there were no detectable change in ICAM-1 above baseline constitutive expression. VEGF and TNF-α/LPS activation of HEMEC served as negative and positive controls, respectively. Results are representative of 3 independent experiments. B: inhibition of both Akt and p38 MAPK pathways in siRNA-transfected HEMEC resulted in VCAM-1 inhibition following acidic activation. C: whole cell radioimmunoassay demonstrates the differential VCAM-1 surface expression in response to pH 4.5 between human intestinal microvascular cell (HIMEC) and HEMEC monolayers. Representative results from 3 independent experiments are shown.
decreased apoptosis, which corresponded with activation of p38 MAPK, activation of ERK, and a delayed activation of JNK. In these studies, increased epithelial proliferation in response to acidic pH was abolished by inhibition of p38 MAPK or JNK, while inhibition of apoptosis was linked to ERK activity. Souza correlated these in vitro studies with biopsies from Barrett’s esophagus patients, taken before and after of esophageal acid perfusion.

In parallel with these findings in esophageal epithelial cells, our studies in esophageal endothelial cells also demonstrate a significant activation response to acid exposure characterized by increases in p38 MAPK activity at 60 min, and a transient rapid increase in JNK activity at 5 min along with Hsp27 and Hsp70 expression, which was controlled at least in part by protein kinases. Thus activation of MAPK signaling pathways appears to play a central role in HEMEC activation in response to acidic pH stimulation. However, the relationship of Hsp27 and Hsp70 to the activation of these protein kinase signaling pathways following esophageal endothelial cell activation by acidic pH is unknown.

It is now appreciated that the MAPK family plays an important role in coordinating gene responses to various stresses, which will include induction of Hsps. Specific inhibitors of p38 MAPK (SB-203580) and p44/42 MAPK (PD-098059) are known to eliminate Hsp70 induction in various cell types (24). It has been shown that phosphorylation and
activation of Hsp27, a substrate for p38 MAPK, is present in chronically hypoxic infant hearts but not in normoxic hearts (24). Overexpression of Hsp27 in myocytes confers protection against simulated ischemia (39). On the basis of this rationale, we reasoned that Hsps might also play a role in esophagitis, where local esophageal cell populations are subjected to the chronic stress of acidic pH exposure.

Hsp27 is a small Hsp that is believed to function as a microfilament capping protein in vitro (42) and is known to be constitutively expressed at high levels in the lung (29). In response to stress, Hsp27 undergoes rapid phosphorylation, which results in actin polymerization and stress-fiber formation (21, 35). The 70-kDa family of Hsps (Hsp70) plays a vital role in cellular protection and has been detected in essentially all tissues subjected to acute and chronic stress (3, 4). The Hsp70 stress proteins exist as two isoforms in eukaryotic and prokaryotic cells, specifically the inducible form (Hsp70) and constitutive form (Hsc70). Messenger RNA and protein for the cognate form of Hsc70 are constitutively expressed in non-stressed cells. The Hsc70 isoform may increase expression and functions primarily as a molecular chaperone. In contrast, Hsp70 is highly inducible and upregulates in response to stressful stimuli, where it will also function as a molecular chaperone. Overexpression of mRNA for Hsp70 using gene therapy resulted in increased protein levels that were associated with cardioprotection in hypoxic rabbit hearts, suggesting that this stress protein plays an important role in mediating resistance to myocardial ischemia (2, 34, 48). In endothelial populations, Hsp70 and Hsp27 have also been implicated in the protection of cells against apoptosis (26). However, the role of Hsps in esophagitis and esophageal endothelial activation has not been defined.

**Fig. 10.** Akt activation in HEMEC in response to acidic pH. **A**: Western blot and densitometric analysis of prolonged acidic pH-activated HEMEC lysates demonstrated activation of Akt/PI3K. Akt phosphorylation by acidic pH exposure in HEMEC was inhibited by PI3K inhibitor (LY-294002, 10 μM). Nonphosphorylated Akt demonstrates equivalent amounts of protein loading for each condition. Results are representative of 3 independent experiments. **B**: HEMECs transfected with Akt siRNA did not express Akt following acidic exposure. **C**: pretreatment of cells with 2 μM 17-AAG for 30 min before acidic pH activation resulted in decreases expression of Hsp70 in HEMEC.
Pretreatment of HEMEC with pharmacological inhibitors of protein kinases for 30 min before acidic exposure resulted in reduced expression of Hsp27 and Hsp70 message and protein. These findings were confirmed using an siRNA gene silencing strategy inhibiting p38 MAPK as wellAkt. Inhibition of protein kinases had no effect on expression of Hsc70 mRNA and protein in HEMEC. SB-203580, a selective p38 MAPK inhibitor, as well as p38 MAPK siRNA transfection, abolished both Hsp27 and Hsp70 message and protein in HEMEC exposed to acidic pH but not in unstimulated control cells. These findings suggest that p38 MAPK activity is essential for acid-induced upregulation of Hsp27 and Hsp70, which may contribute to subsequent cytoprotection. Our observation confirms previous findings in chronically hypoxic astrocytes where SB-203580 attenuated the increase in Hsp70 message (57). Furthermore, hypertonic induction of Hsp70 message in the kidney is inhibited by SB-203580 (53). These findings suggest p38 MAPK activation is essential for adaptation to stress by induction of heat shock proteins. However, inhibition of Hsp70 by SB-203580 may be the result of inhibition of HSF1 phosphorylation, which we also identified in esophageal endothelial.
The effect of staurosporine, a potent PKC inhibitor, on HSF-HSE binding indirectly by heat in HT-29 cells was examined by Erdos and Lee (16). They demonstrated that staurosporine treatment did not alter heat-induced HSF-HSE-binding ability and concluded that staurosporine did not inhibit HSF1 phosphorylation. However, other authors have demonstrated that staurosporine suppresses the accumulation of Hsp70 mRNA in HT-29 cells induced by heat. They reasoned that Hsp70 mRNA suppression was the result of decrease in initiation and elongation activity of the Hsp70 gene. Similarly, general inhibitors of PKC, PKA, and PKG (e.g., H-7 and H-8) have been shown to suppress heat-induced accumulation of Hsp70 mRNA (16). These data suggest that protein kinases contribute to the synthesis of Hsp70 mRNA via HSF1 phosphorylation without showing which protein kinase was involved. In addition to protein kinases, other genes have been shown to be important in regulating Hsp70 expression in response to stress. In an elegant study by Zhao et al. (60), the role of the double-stranded RNA-dependent protein kinase gene (pkr) was shown to be essential in the heat stress response and was also involved in regulating expression of Hsp70 and other heat shock proteins through mRNA stabilization. Finally, there are numerous reports clearly demonstrating the cytoprotective role of Hsp70 in vitro by means of heat shock induction or Hsp70 overexpression (25, 36, 38) in response to toxicity induced by several cytokines.

We explored the intracellular signaling mechanisms that were linked to enhanced survival of HEMEC exposed to acidic pH. In addition to the MAPK activation described above, PI3K/Akt activity played an important role in HEMEC survival in the presence of acid. Akt is a serine threonine kinase, which functions in endothelial cells to promote survival independent of matrix attachment, acting downstream of growth factors. Recent reports have linked Akt signaling in endothelial cell stress responses through modulation of Hsp70 (28). Kim et al. (28) found that Hsp70 is a new antiapoptotic target of Akt-FOXO3a signaling in human umbilical vein endothelial cells. Hsp70 controlled endothelial viability through modulation of the stress induced intrinsic cell death pathway. Our findings confirmed a link between acid induced activation of HEMEC and enhanced cell survival through Hsp70, which also correlated with Akt activation.

Our data demonstrate that acidic pH stress in HEMEC resulted in increased Hsp27 and Hsp70 expression at the level of mRNA and protein. Following stress, the principal response of the cell is rapid and transient reprogramming of cellular activity with an increase in the synthesis of regulatory proteins. It has been shown that reduction of Hsp concentration by Hsp binding to damaged, degraded or abnormal proteins induces Hsp synthesis (9). Acidic pH-induced stress-fiber formation in HEMEC. This is in agreement with the presumed role of Hsp27 proteins which are considered “nurse” molecules able to prevent and reverse the denaturation of various proteins fundamental to normal cellular activity (8, 41, 59). Furthermore, recent data suggest that reorganization of the actin cytoskeleton, a key function of the Hsp’s during stress, has been associated with changes in endothelial permeability, endothelial motility (i.e., an early component of angiogenesis) as well as increased endothelial adherence for leukocytes (6, 27). We demonstrated that acidic pH-induced Hsp 70 expression in HEMEC exerted a protective effect on HEMEC survival, which corresponded with decreased apoptosis. The effect of acidic pH was almost as potent as thermal stress in HEMEC survival in our assay system. These data suggest that esophageal microvascular endothelial cells exposed to acidic pH will mount a stress response that will preserve the cells through the induction of an acid-induced heat shock response. Although the entire functional significance of increased Hsp expression in HEMECs was not addressed in this work, the adaptation of this endothelial population to chronic stress will likely be paralleled with additional alterations in tissue specific microvascular physiology, which will be the subject of future studies.

REFERENCES