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Analysis of Mucosal Stress Response in Acid-Induced Esophagitis in Opossum

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This study is the first to examine site-specific changes in mucosal antioxidants and expression and localization of heat shock proteins (HSPs) following the induction of subacute esophagitis and after recovery using an established animal model. Distal, middle, and proximal samples were excised from anesthetized opossums 24 hr after three consecutive days of 45-min perfusion with saline or 100 mmol/liter HCl, or seven days after acid in recovery animals. Compared to controls, acid-induced erosive esophagitis significantly increased glutathione peroxidase and HSP90 at all sites and HSP60 proximally. Reduced glutathione was significantly decreased distally, as was HSP72 at distal and middle sites. No changes in superoxide dismutase or catalase occurred. After recovery, superoxide dismutase, catalase, and HSP expression were not different from controls. Glutathione peroxidase and glutathione were significantly decreased distally. Similar differential stress responses may occur in patients with chronic gastroesophageal reflux and could be important in the pathogenesis of reflux esophagitis.

KEY WORDS: heat shock protein; antioxidant; free radical; reflux esophagitis; animal model.

Oxygen-derived free radicals are highly reactive molecules produced during many normal biological processes (1). Their synthesis is markedly increased, however, as part of the inflammatory response, and they are believed to contribute to tissue injury in the absence of sufficient antioxidant defenses (2). Superoxide dismutase (SOD) catalyzes the conversion of superoxide (O_2^-) to hydrogen peroxide (H_2O_2), which is converted to water and oxygen by catalase (CAT). Glutathione peroxidase (GPx) also reduces H_2O_2, but does so through the concomitant oxidation of reduced glutathione (GSH), itself an important antioxidant (1, 3).

Gastrointestinal mucosal injury caused by hemorrhagic shock (4), ethanol (5), ischemia–reperfusion (I/R) (6), and nonsteroidal antiinflammatory drugs (NSAIDs) (7) have all been linked to increased free radical formation in animal models. A reduced capacity of antioxidant defenses is thought to directly contribute to trinitrobenzene sulfonic acid (TNBS) -induced colonic lesions in rats (8) and to inflammatory bowel disease (IBD) in humans (9, 10). Free radicals have also been implicated in acute models (exposure period of 2–24 hours) of reflux esophagitis in rats (11, 12) and rabbits (13), while the administration of antioxidants is protective in these animals (14). Recent studies in rabbits have demonstrated an important role for O_2^- in high-grade esophagitis, but not low-grade esophagitis, both of which were induced by

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repeated luminal exposures of different durations to acid and pepsin over periods of five to seven days (15, 16). Furthermore, increased levels of free radicals have been found in patients with esophagitis and with Barrett's esophagus (17).

There is growing evidence to suggest that oxidative stress is linked to changes in the expression of heat shock proteins (HSPs) (18–20). Under normal conditions, HSPs function as molecular chaperones that prevent protein aggregation and assist newly translated proteins in achieving their appropriate tertiary structure. They can be induced to higher levels of expression during cell division and growth, and after exposure to a variety of stresses, such as heat, heavy metals, amino acids, ischemia, microbial infection, drugs, ethanol, and malignant transformation (18, 21, 22). In addition to acting as chaperones, HSPs in stressed cells act to repair and restore function to renatured proteins and to eliminate irreversibly damaged or inappropriately activated proteins via degradative pathways (22, 23).

The importance of HSPs in mucosal defense is highlighted by an extensive body of animal research. Protection against acetic acid-induced colitis has been afforded by preinduction of HSP72 and HSP90, but not by increased HSP60 (24). In the small intestine, HSP72 is protective against I/R-induced injury (25), while HSP60 is not protective against acetic acid-induced lesions (26). Interestingly, HSP72 protects against NSAID-induced gastric mucosal damage, but has no effect on similar injury in the small intestine (27, 28). In humans, enhanced intestinal expression of HSP60, but not HSP90, is associated with IBD (29, 30). These patients also display an increased expression of HSP72 that is independent of the degree of inflammation (31).

However, very little attention has been paid to the expression of HSPs in the normal esophagus and in patients with reflux esophagitis, and no studies of the importance of stress proteins in esophageal disease have been carried out in animal models. The limited data available indicate that high levels of HSP27 are found in the human esophagus, while its expression is markedly decreased in individuals with Barrett's esophagus and adenocarcinoma (32). HSP60 and HSP72 are present constitutively in rat esophageal cells and HSP72 expression is decreased after exposure to heat stress in humans (2, 33).

Therefore, the purpose of this investigation was to determine the effects of repeated esophageal acid exposure on two important components of mucosal defense in an established subacute animal model: the antioxidant status and the expressions of HSP60, HSP72, and HSP90. Changes in antioxidant and HSP levels following tissue recovery from esophagitis were also examined. In addition, the cellular localizations of each HSP in the normal, inflamed, and recovered esophagus were studied. The opossum was used in this investigation due to the many similarities that its esophagus shares with that of humans, including the presence of submucosal glands; a nonkeratinized, stratified, squamous epithelium; and an analogous distribution of striated and smooth muscle (34, 35).

MATERIALS AND METHODS

Animal Preparation. The protocol was approved by the Queen's University Animal Care Committee in accordance with guidelines established by the Canadian Council on Animal Care. Adult opossums (*Didelphis virginiana*) of either sex, weighing 2.1–5.8 kg, were fasted for 8–12 hr prior to each day of experimentation, but were allowed free access to water. Anesthesia was induced each day via tail vein injection (intravenously) with ketamine hydrochloride (25 mg/kg) and diazepam (Valium; Hoffman-La Roche Ltd., Mississauga, Ontario, Canada) (1 mg/kg). Buprenorphrine (Buprene; Reckitt & Colman Pharma Inc., Richmond, Virginia, USA) (0.01 mg/kg) was given intravenously to maintain analgesia. Animals were secured supine to a heated surgery table at a 30° head-up angle to minimize reflux of the perfusate. Body temperature was kept at 35°C with a heating pad. An endotrachial tube was placed, and the cuff was inflated to minimize aspiration. At the end of days 1–3, 100 ml of lactated Ringer's solution was administered subcutaneously to maintain fluid and electrolyte balance, and 0.05 mg/kg atropine sulfate (Abbott Laboratories Ltd., Montreal, Quebec, Canada) was given intravenously to prevent vomiting and excessive secretion.

Experimental Protocol. Manometric localization of the lower esophageal sphincter (LES) was accomplished using a custom-designed opossum LES Sleeve Assembly catheter system (Dentsleeve; Pty Ltd., Bowden, South Australia). A second catheter for esophageal luminal perfusion was passed orally and positioned 7.0 cm proximal to the manometrically determined LES. Animals were given a 30-min stabilization period and were subsequently perfused for 45 min on each of three consecutive days with either 0.9% saline (NS) (control) or 100 mmol/liter hydrochloric acid (HCl), which was made isosmolar with NS. Twenty-four hours later (day 4), the esophagus was excised and animals were sacrificed with an overdose of pentobarbital sodium (Somnotol; MTC Pharmaceuticals, Cambridge, Ontario, Canada). An additional group of "recovery" animals was used to explore the resolution phase of injury. These animals were perfused with acid as above, but were then allowed to recover for seven days prior to sacrifice and tissue removal. All perfused solutions were prewarmed to 37°C and administered at a rate of 2.0 ml/min using a peristaltic cassette pump (Manostat, New York, New York).

In all animals, the excised esophagus was rinsed thoroughly, immersed in ice-cold NS solution, opened longitu-

dinally, then pinned out, mucosal side up in a wax dish. Transverse tissue sections were taken from distal, middle, and proximal regions of the esophagus (1–3, 5–7, and 9–11 cm proximal to the gastroesophageal junction (GEJ), respectively, for immunohistochemistry, histology, antioxidant enzyme assays, and western blotting; 0–1, 4–5, and 8–9 cm, respectively, for assay of myeloperoxidase (MPO) activity, an index of inflammation). The mucosa and submucosa were removed, using blunt dissection, from samples taken for western blotting and antioxidant and MPO assays, blotted dry, immediately snap-frozen in liquid nitrogen and stored at −80°C until use. Histology and immunohistochemistry samples were immersion-fixed in Carnoy's fixative (10% glacial acetic acid, 30% chloroform, 60% absolute ethanol, by volume) for 2–4 hr, then stored in 70% ethanol. After routine histologic processing and embedding in paraffin, samples were sectioned (6 μm) and mounted on glass slides.

Measurement of MPO Activity and Epithelial Injury. MPO activity was assayed using the method of Krawisz et al. (36), with modifications described by Schierwagen et al (37). Sections processed for routine histology were stained with hematoxylin and eosin and then examined by a blinded observer. The percent epithelial denudation (complete absence of epithelium) was calculated as: % denudation = (total length of all denuded portions/total length of section) $\times$100.

Antioxidant Assays. Distal, middle, and proximal samples were powdered under liquid nitrogen and homogenized in 20 vol of ice-cold homogenization buffer (20 mM HEPES (pH 7.0), 1 mM EDTA and 0.1% Triton X-100). Homogenates were then used to measure SOD, CAT, and GPx activities using the methods of McCord and Fridovich (38), Aebi (39), and Flohé and Gunzler (40), respectively. In distal samples only, GSH content was determined by measuring the total soluble sulfhydryl concentration according to a method adapted from Wakulich and Tepperman (41).

Immunohistochemistry. Following processing and mounting on glass slides, sections were treated with 3% H_2O_2 in deionized water for 30 min to quench endogenous peroxidase activity, then incubated with 5% bovine serum albumin (BSA) for 1 hr to block nonspecific antibody binding. Sections were then incubated overnight with primary antibodies against HSP60 (1:200; SPA 804, which is specific for HSP60), HSP72 (SPA 810, which is specific for the inducible HSP72 and does not react with the constitutive HSP73), or HSP90 (SPA 835 which detects both alpha and beta isoforms of HSP90 equally well). Antibodies were obtained from StressGen Biotechnologies Co., Victoria, British Columbia, Canada.

For the detection of bound antibodies to HSP60 and HSP72, sections were washed with 50 mM Tris-buffered saline (TBS), pH 7.6, and subsequently incubated with biotinylated immunoglobulin G (IgG) (1:300; anti-rabbit IgG for HSP60; anti-mouse IgG for HSP 72; Dako). After washing again, sections were incubated with horseradish peroxidase-conjugated streptavidin (1:500; Dako). For localization of HSP90, sections were incubated with horseradish peroxidase-conjugated anti-rat IgG (1:400; StressGen). Peroxidase activity was visualized by incubating sections for 15 min in TBS containing 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.03% H_2O_2.

Negative controls for each HSP were treated as above with omission of the primary or secondary antibody or use of "mismatched" secondary antibodies (eg, anti-rabbit IgG was used instead of anti-mouse IgG for HSP72 detection). No signal was observed for any HSP with any of these procedures. All antibodies were diluted in TBS and all incubations were carried out at room temperature in a humidified chamber for 1 hr, unless otherwise specified.

Western Blotting. Samples were chopped finely with scissors and homogenized using 5 vol of ice-cold lysis buffer (50 mM Tris HCl, pH 8.8, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1.0% Triton X-100, 0.5 mM PMSF, and 1 μg/ml leupeptin). Homogenates were centrifuged at 27,000*g* for 15 min at 4°C and supernatant aliquots were stored at −80°C. The protein content of each supernatant was determined using the bicinchoninic acid method (42). After mixing with an equal volume of sample buffer and boiling for 4 min, samples (20, 10, and 30 μg protein/lane for HSP60, HSP72, and HSP90 determinations, respectively) were separated by electrophoresis using 8% SDS-polyacrylamide gels and then transferred to polyvinyldiene difluoride (PVDF) membranes (Bio-Rad Laboratories, Mississauga, Ontario, Canada).

Overnight incubation of the membrane in blocking solution [5% nonfat dried milk in TBS containing 0.02% Tween-20 (TBST)] was carried out at 4°C to reduce nonspecific antibody binding. This was followed by membrane incubation with primary antibody [rabbit anti-HSP 60 (1:1000 for 1 hr), mouse anti-HSP 72 (1:2000 for 1 hr) or rat anti-HSP 90 (1:250 for 2.5 hr) (StressGen)]. After washing the membrane with TBST, primary antibodies were detected by 1-hr incubation with alkaline phosphatase-conjugated anti-rabbit (1:3000), anti-mouse (1:3000), or anti-rat (1:1000) IgG (Sigma Chemical Co., Oakville, Ontario, Canada), after which washing was repeated. Alkaline phosphatase activity was then developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma) as the substrate. All antibodies were diluted in blocking solution, and all antibody incubations and washes were carried out with gentle agitation at room temperature.

Negative controls for each HSP were treated as above, except that mismatched secondary antibodies were used. No signal was observed for any HSP with this procedure. Two lanes of each gel served as positive controls and were loaded with heat-shocked HeLa cell lysate (StressGen; 5 μg/lane for detection with anti-HSP60 and anti-HSP72; 20 μg/lane for anti-HSP90). Optimal antibody concentrations and the amount of sample protein and HeLa cell lysate loaded for detection of each HSP were determined from preliminary studies.

After being allowed to air dry, video densitometry (Image-Pro® Plus 3.0, Media Cybernetics, Silver Springs, Maryland, USA) was used to measure the integrated optical density (product of area and density) of each band. The relative density of each sample band was determined using the following formula: relative density (%) = (density of sample band/mean density of HeLa cell lysate bands) $\times$100.

Statistics. Data are presented as means $\pm$ SE. All statistics were performed on an IBM PC-compatible computer using Excel (Microsoft Corp., Redmond, Washington, USA) and Instat (Graphpad Software, San Diego, Califor-

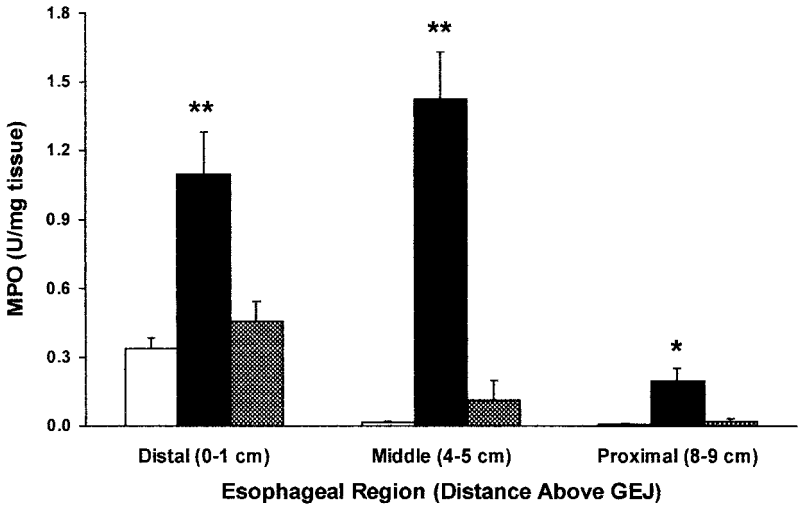

Fig 1. Effect of repeated esophageal luminal perfusion of 0.9% saline, 100 mmol/liter HCl, or repeated perfusion of acid followed by seven days of recovery on mucosal MPO activity. Compared to saline-perfused controls ($N = 14, 14, 6$ for distal, middle, and proximal esophageal sites, respectively), repeated acid exposure ($N = 14, 15, 6$) caused significant increases in MPO activity at all sites. Recovery animals ($N = 4, 4, 4$) were not significantly different from controls at any site ($^*P < 0.01$; $^{**}P < 0.001$; open bars = saline; closed bars = acid; hatched bars = recovery).

nia, USA) analytical software. Differences in MPO, SOD, CAT, GPx, GSH, and the expressions of HSP60, HSP72, and HSP90 at each esophageal site between NS-perfused controls, acid-perfused, and recovery groups of animals were tested for statistical significance using one-way analysis of variance (ANOVA) with Bonferroni *post-hoc* tests comparing controls to acid and recovery animals. Differences in percent epithelial denudation at each site between the three groups were tested for statistical significance using the Kruskal-Wallis test with Dunn's multiple comparisons *post-hoc* tests comparing controls to acid and recovery animals. In controls, regional differences in MPO, SOD, CAT, GPx and the expressions of each HSP were tested for statistical significance using repeated measures ANOVA with Tukey *post-hoc* tests to determine which sites differed. Differences in the expressions of each HSP at the distal site between unperfused and NS-perfused animals were tested for statistical significance using two-tailed Student's *t* tests. $P < 0.05$ was considered statistically significant.

RESULTS

MPO Activity and Epithelial Injury

In NS-perfused control animals, MPO activity was significantly greater in the distal esophagus than in the middle and proximal regions, while no difference was found between the latter two sites (Figure 1). Macroscopically, repeated acid perfusion induced mucosal sloughing, reactive hyperemia, necrosis, and ulceration, all of which were absent in recovery animals. This was associated with significant increases in epithelial denudation at the distal and middle sites (Table 1) and in MPO activity at all sites (Figure 1). The potential impact of the considerable acid-induced epithelial cell loss on the accurate measurement of mucosal antioxidants and HSPs (see below) was not assessed. In recovery animals, no differences from controls in epithelial denudation or MPO activity were found at any site.

Mucosal Antioxidant Levels

In controls, distal SOD and GPx activities were each significantly greater than the corresponding activities in middle and proximal samples. GPx at the

Table 1. Effect of Repeated Esophageal Intraluminal Perfusion of 0.9% Saline (NS) or Acid or Repeated Perfusion of Acid Followed by Seven Days of Recovery on Epithelial Denudation*

	NS	*Acid*	*Recovery*
Proximal	3.7 ± 2.2	0.0 ± 0.0	0.0 ± 0.0
Middle	0.5 ± 0.5	22.4 ± 9.2[a]	0.0 ± 0.0
Distal	2.3 ± 1.4	30.1 ± 8.4[b]	10.4 ± 4.2

*Acid was 100 mmol/liter HCl. Values represent means ± SE for percent epithelial denudation at distal, middle, and proximal esophageal sites (1–3, 5–7, and 9–11 cm above the gastroesophageal junction, respectively). Compared to NS-perfused controls ($N = 13, 13$, and 6 for distal, middle, and proximal sites, respectively), repeated acid perfusion (N = 13, 13, and 6) caused significant denudation at the distal and middle sites, but not at the proximal site. Recovery animals ($N = 4, 4$, and 4) were not significantly different from controls at any site. [a]$P < 0.01$; and [b]$P < 0.001$.

middle site was significantly greater than was observed proximally, while no difference in SOD activity was found between these sites (Figure 2A,C). No significant regional differences in CAT activity were found (Figure 2B). Repeated acid perfusion did not significantly affect SOD or CAT at any site (Figure 2A,B). In contrast, GPx activity was significantly increased at all sites, while a significant decrease in GSH content was found distally. Recovery animals exhibited GPx levels that were not significantly different from controls at the middle or proximal sites, but distal GPx activity and GSH content were significantly reduced (Figures 2C and 3). No changes in SOD or CAT were observed after recovery (Figure 2A,B).

Localization of HSPs

Epithelium. The distal esophagus was characterized by a highly convoluted mucosa covered by a thin epithelium. All three HSPs were detected only in basal cells in NS-perfused controls (Figure 4A,D,G). In areas not denuded of epithelium by acid exposure, increased staining for both HSP60 and HSP90 were noted throughout, including in superficial cells (Figure 4B,H), while HSP72 immunoreactivity was markedly decreased and often absent in all layers (Figure 4E). Recovery animals displayed similar distributions and intensities of HSP staining to those found in controls, although a much thicker epithelium was often observed (Figure 4C,F,I).

A markedly thicker epithelium containing readily differentiated cuboidal cells of the stratum germinativum (SG) and flatter squamous cells of the stratum spinosum (SS) and stratum corneum (SC) was apparent at the middle site (Figure 5). Basal cell hyperplasia induced by repeated acid exposure was detected in many nondenuded areas, while this was not observed after recovery. In all animals, HSP60 displayed granular, nonnuclear staining, while HSP72 and HSP90 exhibited more diffuse cytosolic staining. In controls, pronounced HSP60 staining was seen along the basolateral surface of cells of the SG (Figure 5B). Strong immunoreactivity was also evident throughout the SS, especially in the lower half, where a band of stain extended laterally outward from the nucleus. HSP72 staining was most intense in the SG, but was also found in the lower half of the SS (Figure 5E). The SC did not contain HSP60 or HSP72. The strongest HSP90 staining was noted in the SG and SS, with staining also found in the SC (Figure 5H).

After repeated acid perfusion, HSP60 was found in all epithelial layers, including the SC, with a gradient of decreasing intensity towards the lumen (Figure 5C). In contrast, the SG and SC did not contain HSP72, while the entire SS was positive (Figure 5F). Although acid challenge did not change the distribution of HSP90, the intensity of staining was enhanced, especially in the lower half of the epithelium (Figure 5I). In recovery animals, a similar epithelial distribution of HSP60, HSP72, and HSP90 to that of controls was noted (not shown).

The epithelial distribution of all three HSPs at the proximal site (not shown) closely resembled that of the middle site in all animals, with a few minor exceptions. After acid exposure, no HSP60 staining was observed in the most superficial layers and only some cells of the SG were negative for HSP72. In addition, the intensity of HSP72 immunoreactivity was decreased in recovery animals, relative to NS-perfused controls, despite a similar distribution.

Lamina Propria, Submucosa, Muscularis Mucosa, and Other Muscle Layers. Pronounced HSP90 staining was observed throughout the lamina propria and submucosa at all sites in all animals, while no HSP60 or HSP72 staining was found (Figures 4 and 5). In all animals, no staining for any HSP was detected in the muscularis mucosa or in the circular or longitudinal muscle layers at any site.

Esophageal Glands. In the distal esophagus, glands were located within the lamina propria (Figure 4), while they were found in the submucosa, and in much fewer numbers, in the middle and proximal regions (Figure 5A,D,G). In controls, granular, nonnuclear HSP60 staining was observed in cells around the outer edges of the glands, especially at the distal site (Figure 4A). After acid challenge, many glands had released their contents and a decrease in HSP60 staining was noted (Figure 4B). In recovery animals, HSP60 immunoreactivity was similar to that seen in controls (Figure 4C). In all animals, no staining for HSP72 or HSP90 was observed at any site in cells surrounding the esophageal glands.

Mucosal Expression of HSPs

In control animals, HSP60 expression detected by western blot at the middle site was significantly greater than at the proximal site (Figure 6A). In contrast, HSP72 content in the middle region was significantly greater than in the distal region (Figure 6B). HSP90 was found to be significantly greater distally than in either middle or proximal samples. No other significant differences between sites were detected (Figure 6C). An additional group of four animals was studied to exclude the possibility that re-

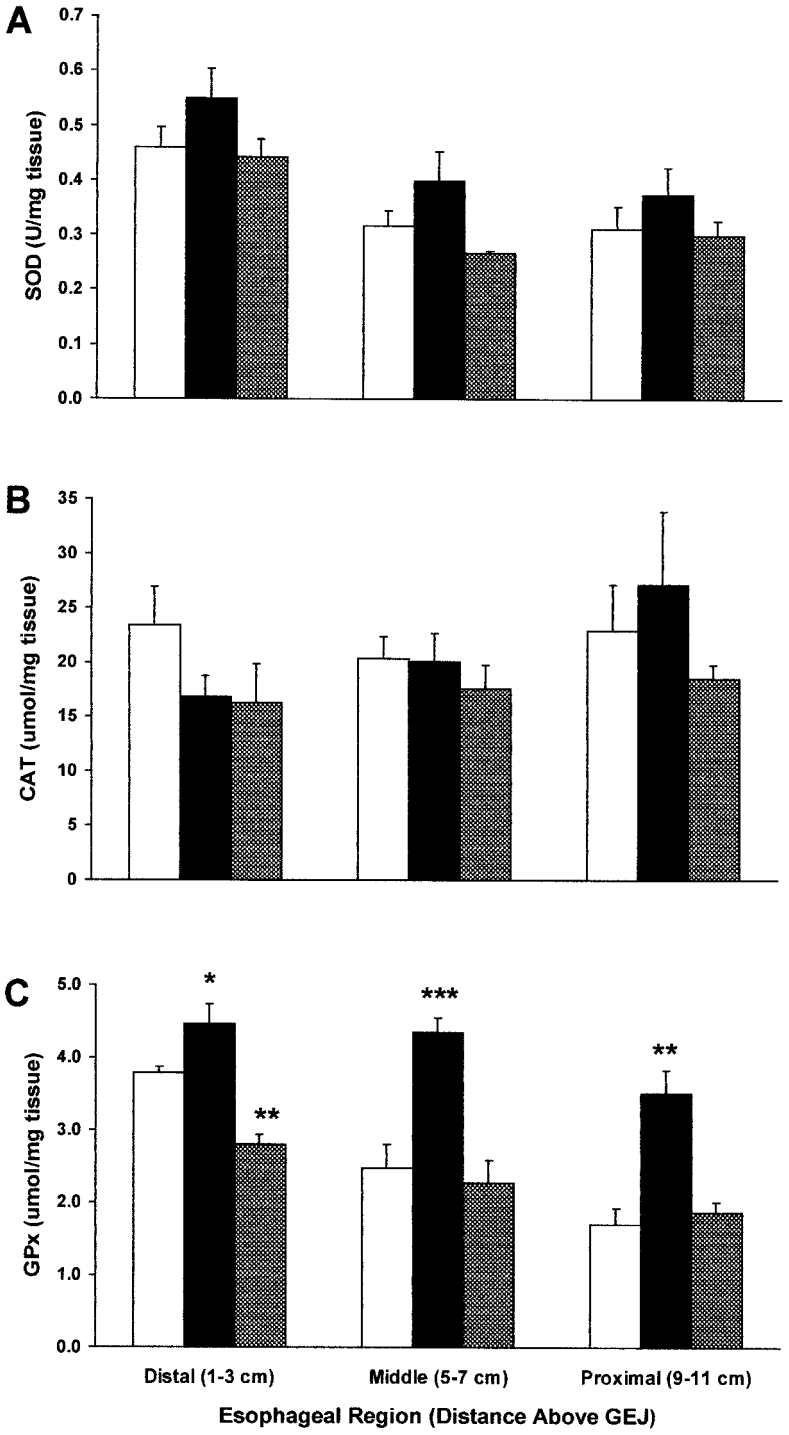

Fig 2. Effect of repeated esophageal luminal perfusion of 0.9% saline, 100 mmol/liter HCl, or repeated perfusion of acid followed by seven days of recovery on mucosal antioxidant enzyme levels. No significant differences from saline-perfused controls ($N = 6, 6, 6$ for distal, middle, and proximal esophageal sites, respectively) were found in SOD (A) or CAT (B) activities at any site. Acid perfusion ($N = 6, 6, 6$) induced significant increases in GPx levels at each site (C). In recovery animals ($N = 4, 4, 4$), no significant differences from controls were noted in SOD or CAT activities at any site or in GPx content at the middle or proximal sites. However, GPx was significantly decreased distally ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; open bars = saline; closed bars = acid; hatched bars = recovery).

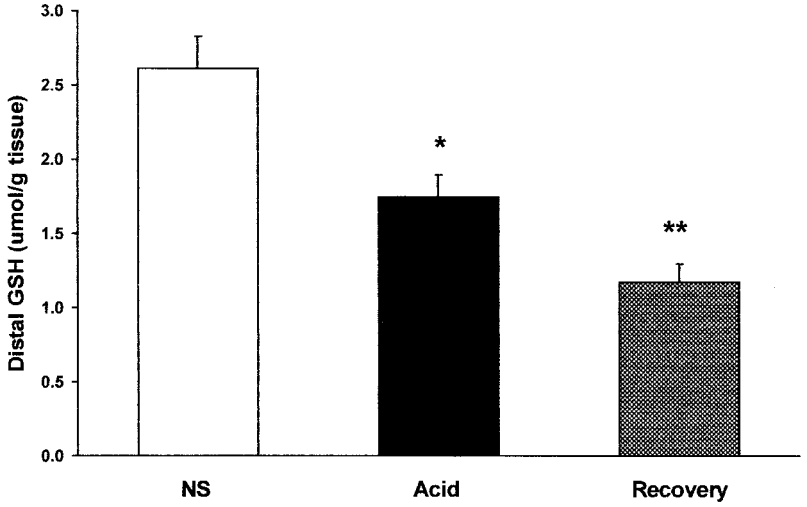

Fig 3. Effect of repeated esophageal luminal perfusion of 0.9% saline, 100 mmol/liter HCl, or repeated perfusion of acid followed by seven days of recovery on mucosal GSH content at the distal site (1–3 cm above the GEJ). Compared to saline-perfused controls ($N = 7$), repeated acid exposure ($N = 9$) induced a significant decrease in GSH content. GSH was also significantly decreased after seven days of recovery ($N = 4$) ($^*P < 0.01$; $^{**}P < 0.001$).

peated anesthesia and handling affected basal HSP expression. Distal mucosal samples were removed immediately after anesthesia on day 1 from unperfused animals. No significant differences in HSP expression were found, compared to distal samples taken from NS-perfused animals (66.5 ± 10.2% relative density vs 62.8 ± 5.7%, 108.0 ± 12.6% vs 98.5 ± 4.1%, and 58.0 ± 6.7% vs 49.6 ± 2.8% for unperfused vs NS-perfused animals for HSP60, HSP72, and HSP90, respectively).

Repeated exposure to acid induced a significant increase in HSP60 expression at the proximal site, but not at the distal or middle sites (Figure 6A). On the other hand, HSP72 expression was significantly decreased at the distal and middle sites, but was unchanged proximally (Figure 6B). HSP90 expression was significantly increased after acid perfusion at all three sites (Figure 6C). In recovery animals no significant changes in HSP expression were observed at any site, compared to controls.

DISCUSSION

Repeated intraluminal acid perfusion of the opossum esophagus induced significant inflammation at all sites, while marked epithelial denudation was noted in the distal and middle regions only. An observed minor retrograde flow of perfusate likely caused the proximal inflammation, but was not sufficient to cause damage. Recovery from esophagitis and injury were observed at all sites seven days after acid exposure. Similar subacute changes in mucosal inflammation and histology have previously been demonstrated in the cat in both an analogous four-day model of esophagitis and after six months of surgically induced spontaneous gastroesophageal reflux, the latter of which was reversed by acid suppression (43).

In control animals, the greatest MPO activity was detected distally where the close proximity to the stomach would be associated with greater exposure to refluxed gastric contents. Moreover, distal SOD and GPx activities were both found to be significantly elevated compared to the other sites. However, the induction of esophagitis did not significantly affect SOD activity at any site. Consistent with this finding, no changes in O_2^- levels or SOD activity were found after the induction of low-grade esophagitis (mild damage) in rabbits (15). In contrast, patients with reflux esophagitis have been shown to have significantly decreased SOD activity and increased O_2^- production, both of which were directly related to the severity of the inflammation (17). Elevated O_2^- levels have been associated with acute esophageal mucosal injury in rabbits, while exogenous SOD was protective in these animals (13) and in rats 24 hours after duodenojejunal ligation (12,14). Likewise, O_2^- has been implicated in a model of high-grade esophagitis (severe damage) in rabbits, while SOD attenuated this injury (16). Thus, while superoxide is associated

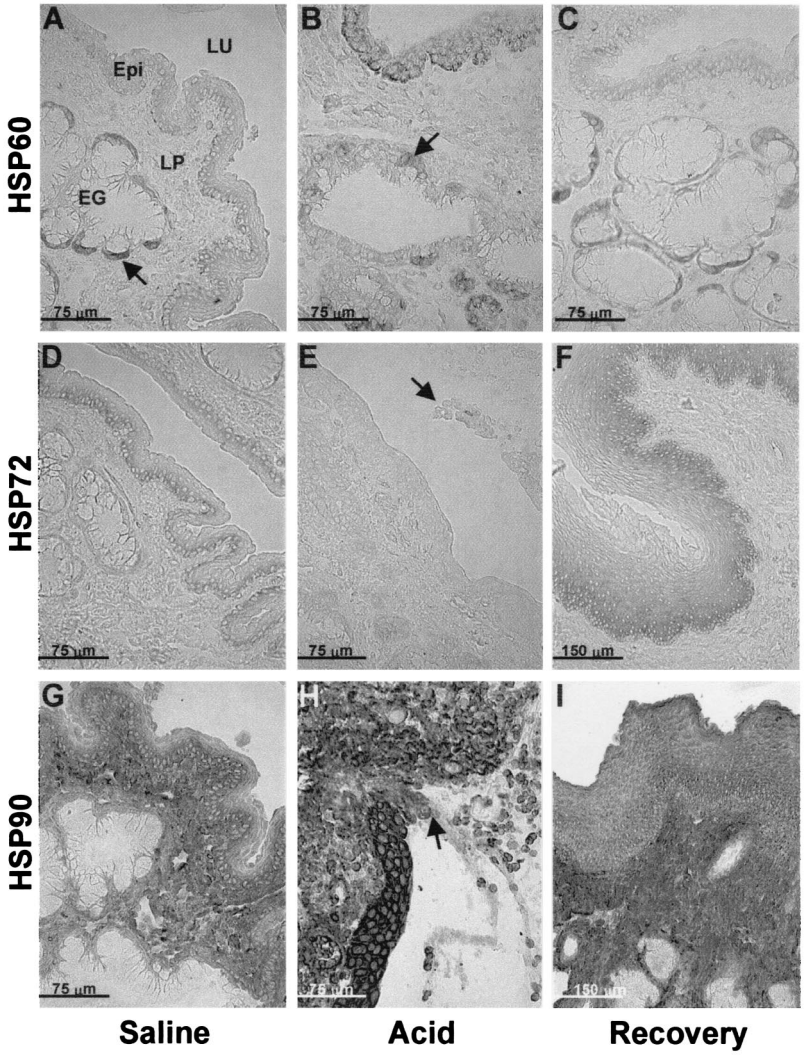

Fig 4. Immunohistochemical localization of HSP60 (A–C), HSP72 (D–F) and HSP90 (G–I) in the distal opossum esophagus (1–3 cm above the GEJ) after repeated luminal perfusion of 0.9% saline (A,D,G), 100 mmol/liter HCl (B,E,H), or repeated perfusion of acid followed by seven days of recovery (C,F,I). (A,B) Arrows indicate HSP60 immunoreactivity in cells surrounding the esophageal glands. (E,H) Arrows indicate damage to the epithelium. (F,I) A marked increase in epithelial thickness was often observed in recovery animals. LU = lumen; Epi = epithelium; LP = lamina propria; EG = esophageal gland.

with the development of injury in these other studies, it did not appear to be important in the present model of esophagitis.

CAT activity was also not significantly affected by acid exposure in this investigation. This is consistent with the finding that CAT administration had no effect on acidified pepsin-induced esophageal damage in rabbits (13). In addition, exogenous SOD and CAT together were not better than SOD alone in preventing damage in a rat model of esophagitis (14). Although CAT was unaffected by acid, the importance of H_2O_2 in the observed injury was highlighted by the significant increases in GPx activity at all three esophageal sites, and the associated significant decrease in distal GSH content. Decreased esophageal GSH has previously been found in rats in the first few hours following duodenojejunal ligation (12). However, GSH levels were significantly elevated from controls 12 hr later, and in another study after one and six weeks (11). This pattern is consistent with negative feedback via the increased activity of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in GSH synthesis (44).

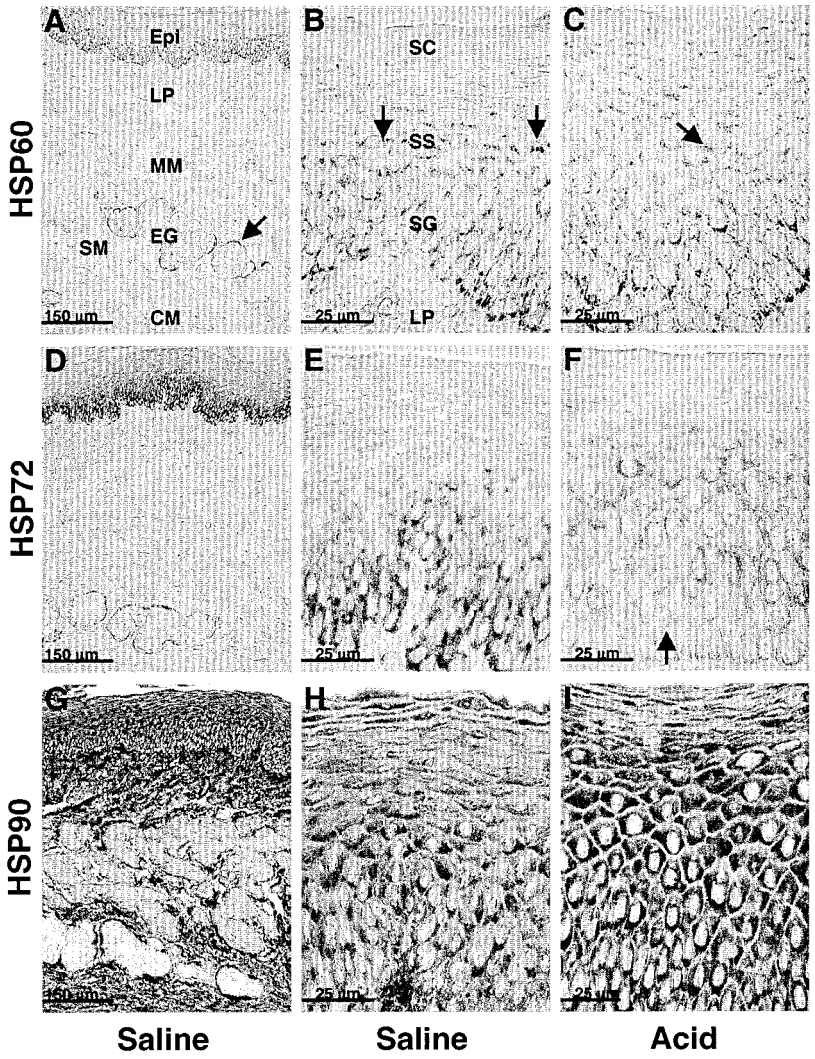

Fig 5. Immunohistochemical localization of HSP60 (A–C), HSP72 (D–F), and HSP90 (G–I) in the middle opossum esophagus (5–7 cm above the GEJ) after repeated luminal perfusion of 0.9% saline (A, B, D, E, G, H) or 100 mmol/liter HCl (C, F, I). (A) arrow indicates HSP60 immunoreactivity in cells surrounding the esophageal glands. (B,C) Arrows indicate a granular, nonnuclear pattern of epithelial HSP60 staining. (F) Arrow indicates a lack of HSP72 immunoreactivity in basal epithelial cells. LP = lamina propria. Epi = epithelium; LP = lamina propria; MM = muscularis mucosa; EG = esophageal gland; SM = submucosa; CM = circular muscle; SC = stratum corneum; SS = stratum spinosum; SG = stratum germinativum.

Interestingly, GSH remained significantly decreased after injury resolution in recovery animals, despite a significant decline from controls in distal GPx activity. This suggests that some component of GSH synthesis, possibly γ-glutamyltransferase (γ-GT) or γ-GCS, was irreversibly inhibited by acid injury or required longer than seven days to recover. Indeed, significant decreases in the activities of both of these enzymes have been associated with a persistent reduction in mucosal GSH in patients with Crohn's disease (10). Furthermore, the potential impact of changes in glutathione reductase and glutathione transferase, which would both impact tissue GSH levels, cannot be overlooked. Given the importance of GSH in the defense against free radicals, such a prolonged decrease would leave the mucosa more susceptible to oxidative injury in the event of additional exposure to acid. The cause of the GPx decrease in recovery animals, and why it occurred only at the distal site while normal levels were at-

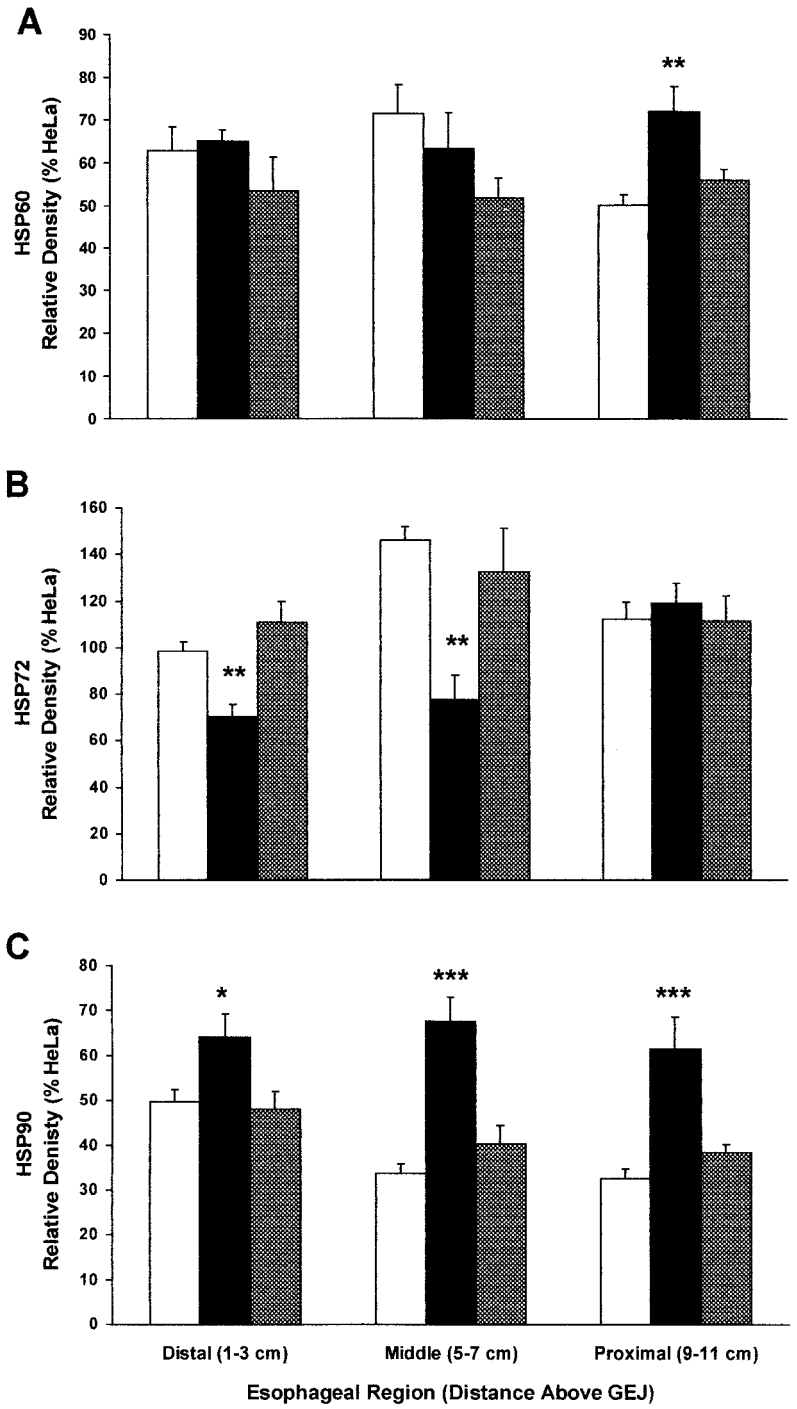

Fig 6. Effect of repeated esophageal luminal perfusion of 0.9% saline, 100 mmol/liter HCl, or repeated perfusion of acid followed by seven days of recovery, on the mucosal expression of HSP60 (A), HSP72 (B), and HSP90 (C). (A) Compared to saline-perfused controls (N = 6, 6, 6 for distal, middle, and proximal esophageal sites, respectively), repeated exposure to acid (N = 6, 6, 6) induced a significant increase in HSP60 expression at the proximal site. (B) HSP72 expression was significantly decreased at the distal and middle sites. (C) HSP90 expression was significantly increased after acid perfusion at all three sites. In recovery animals (N = 4, 4, 4) no significant differences in the expression of any HSP were observed at any site, compared to controls (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; open bars = saline; closed bars = acid; hatched bars = recovery).

tained at the middle and proximal sites, was not explored.

The present subacute investigation is the first to examine in detail, using an established animal model, the effects of acid-induced esophagitis and subsequent recovery on the mucosal expression and localization of the stress proteins HSP60, HSP72, and HSP90. It is not clear if the alterations in antioxidant levels detected in this study were related to the observed changes in HSP expression. Nonetheless, oxidative stress has been linked to the regulation of HSP synthesis in phagocytes (19), in the rat intestinal cell line Caco-2 after hypoxia/reoxygenation (18), and in the postischemic pig liver (20).

HSP60 is primarily a mitochondrial matrix protein that assists in the folding of proteins into their correct tertiary structure (22, 23). In NS-perfused animals, strong HSP60 immunoreactivity was observed in the stratum germinativum and lower stratum spinosum, a pattern similar to that previously described in the rat esophagus (22). This reflects the high rate of replication and protein synthesis characteristic of the basal cells, while the lack of staining in more superficial layers is consistent with senescence of cells as they migrate towards the lumen. Intense HSP60 staining was also observed in cells surrounding the esophageal glands. Although these cells were not identified, it is possible that a group of pyramidal, surfactant-containing cells thought to be involved in active fluid or ion transport to the adjacent gland cells were the source. Electron microscopy has previously shown that these cells contain an abundance of mitochondria, relative to adjacent cells (45).

Repeated acid challenge did not significantly affect HSP60 expression in the distal or middle esophagus. However, basal cell hyperplasia and an increased staining intensity throughout the epithelium, including in the stratum corneum, were found at both sites. In patients with reflux esophagitis, the replication rates of basal cells and their organelles, including mitochondria, are rapidly increased. This leads to luminal cell migration that is faster than the normal rate of senescence, allowing for more rapid epithelial regeneration after injury (46).

HSP72 was found at high levels in the NS-perfused esophagus, especially in the basal epithelium, while acid exposure markedly reduced its expression at the distal and middle sites. As HSP72 is often not detected in unstressed cells (18), these findings imply that its expression may have been previously upregulated in these animals. Decreased HSP72 expression was also detected in human esophageal biopsies after heat shock (33). In contrast to the present study, exposure of these biopsies to acid did not affect HSP72 concentration. However, based on a recent study of the cat esophagus, which established a critical threshold of pH 3.0 for injury to occur (47), the solution used in the human study was likely not acidic enough to induce changes in HSP72 expression (pH 3.65 vs pH $\approx$1.5 in the present study).

It is possible that HSP72 was consumed, but not adequately replenished, as a result of the acid-induced increased rate of basal cell replication and protein synthesis or during the repair of protein damage. Also, a selective degradation of the protein itself or the inhibition of one or more stages of its synthesis, would be consistent with previous findings in lymphoma cells in which decreased HSP72 was attributed to a rapid degradation of heat shock transcription factor (HSF1) (48). It is unlikely that impaired transcription or translation were causes, as 45 min of acid exposure significantly decreased HSP72 expression, but would not have been long enough to induce changes in HSP72 mRNA or protein translation.

HSP90 functions to hold nonnative protein intermediates, primarily those involved in signal transduction, in an unfolded state (49). It is one of the most abundant cytosolic proteins in normal cells and its expression can be induced to higher levels under stressful conditions (23). In this study, HSP90 was observed throughout the lamina propria and submucosa in all animals. As with HSP60 and HSP72, the strongest epithelial immunoreactivity was found in the basal cells, where the highest rates of protein synthesis and need for chaperone activity would occur. In contrast to the other HSPs, however, HSP90 expression was significantly increased at all sites after repeated acid exposure. Enhanced epithelial staining again pointed to increased basal cell replication as an important factor.

In summary, enhanced mucosal GPx activity and decreased GSH content imply that acid-induced esophagitis in the opossum was associated with increased levels of free radicals. Changes in the expression and localization of HSPs were dependent on the HSP in question and on both the esophageal site and cell types involved, suggesting that a different role is played by each HSP in the different cellular compartments. Resolution of inflammation and injury, measured by MPO activity, gross appearance, and histology, was observed seven days after acid exposure. This corresponded with an overall recovery of antioxidant levels and a similar expression and localization of each HSP to those found in controls. However,

cause-and-effect relationships between the acid-induced damage, its subsequent resolution, and the observed changes in mucosal antioxidants and HSPs were not determined. To address this question, future studies will need to be performed on both the protein and mRNA levels to determine if significant temporal changes in mucosal antioxidants or HSPs take place as esophagitis develops or during the restitution phase of injury. Such work would also provide valuable insights into the time course of damage and healing in this model. It would also be desirable to examine how manipulation of one or more of the measured variables would affect the initiation or severity of acid-induced injury and the rate of subsequent recovery. In any case, analogous stress responses may also occur in patients chronically challenged with refluxed gastric contents and could be important in the pathogenesis of gastroesophageal reflux disease.

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