Chronic Acid Water Feeding Protects Mice Against Lethal Gut-derived Sepsis due to *Pseudomonas aeruginosa*

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Abstract

Acidified feeding formulas have been proposed as a method of controlling gastrointestinal colonization and nosocomial infection in critically ill patients. We examined possible mechanisms by which chronic acid water feeding might protect the host against lethal gut derived sepsis by assessing its effect on both local intestinal epithelial barrier function to bacteria as well as on local and systemic heat shock protein expression. Heat shock protein expression measured by immunoblot demonstrated that HSP25 was increased in the stomach, aorta and kidney of mice chronically fed acid water (8 weeks) compared to tap water fed controls. HSP72 expression was also increased in the aorta of mice drinking acid water. The protein content of cecum and its barrier function were enhanced in mice ingesting acidified water. The direct effect of an acid environment on intestinal epithelial barrier function was tested in cultured human intestinal epithelial cells. An acidified environment protected against bacterial mediated disruption of the intestinal epithelial barrier. Finally, the protective effect of chronic acid water feeding on gut-derived sepsis due to *P. aeruginosa* was tested in mice. Chronic acid water feeding protected mice from the lethal gut derived sepsis due to *P. aeruginosa*.

Introduction

It has become increasingly clear that the intestinal tract microflora significantly contribute to the development of various infection-related complications in critically ill patients (Moore, 1999; Souza *et al*., 2004). The microbial contents of the intestinal tract can cause infection-related complications in critically ill patients by contaminating contiguous or remote organs via multiple routes of dissemination. For example, the upper gastrointestinal flora can contaminate the lungs by direct aspiration (Johnson and Hirsch, 2003) while microbes residing in the distal intestinal track can cause infection in remote organs by translocating to tissues when intestinal barrier function is disturbed (Swank and Deitch, 1996). Finally, in some cases the intestinal tract microflora can induce a state of systemic inflammation, often termed gut-derived sepsis, by activating a virulence strategy that dysregulates tight junctional permeability to locally released toxins thereby provoking a potent systemic inflammatory response (Laughlin *et al*., 2000; Wu *et al*., 2003). In recognizing the potential for the intestinal microflora to complicate the course of the critically ill patient, several pre-emptive strategies have been developed to decontaminate patients of their intestinal flora with selective antibiotic decontamination (Kahlke *et al*., 2002). Yet a major concern with this approach is that antibiotic use will lead to the emergence of highly resistant strains of bacterial that will replace the normally protective flora (Schippers and van Dissel, 2003). A second pre-emptive approach that has been described in both animals and humans is acidification of the intestinal microenvironment in an attempt to eliminate all acid sensitive microorganisms (Heres *et al*., 2004). This approach has not gained wide acceptance due to concerns that acidification will lead to the development of systemic acidosis (Carrion and Egan, 1990). In addition, it is unclear how acidification of the intestinal tract by delivery of an acid load (hydrochloric/citric acid) into the stomach or proximal small bowel, will affect the distal intestinal microflora where the greatest microbial burden resides. Although there have been reports that chronic acid water feeding alters the distal intestinal tract microflora, results are not consistent, and the mechanism of this effect is unknown. Finally, whether chronic acid ingestion could affect bacterial mediated disorders of the intestinal tract independent of its direct influence on the intestinal tract microflora has not been previously addressed.

Chronic acid water feeding could indirectly affect the host-pathogen relationship through mechanisms by which acid directly affects host metabolism and immune function. For example, work from the Souba's laboratory has demonstrated that chronic acid water feeding increases the intestinal uptake of glutamine (Epler *et al*., 2003; Pan *et al*., 2004), a conditionally essential amino acid that participates in epithelial cell barrier and immune function. This work has been extended *in vitro* demonstrating that cultured intestinal epithelial cells exposed to an acid environment display marked increases in glutamine uptake (Epler *et al*., 2003). Accelerated uptake of glutamine by the intestinal tract in response to acid water feeding could affect a number of local and systemic cytoprotective processes resulting in enhanced local and systemic immune function. For example, glutamine uptake by intestinal epithelial cells is known to enhance their barrier function by increasing the production of heat shock proteins (Wischmeyer *et al*., 1997). Heat shock proteins are highly conserved across species and are present in a variety of cells. Forced expression of heat shock proteins in intestinal epithelial cells confers enhanced protection and preservation of barrier function against a variety of insults including oxidant injury and bacterial adherence-associated alterations in tight junctional permeability (Musch *et al*., 1999). Therefore given the documented effects of chronic acid water feeding on intestinal glutamine uptake, and the ability of glutamine to up-regulate the production and

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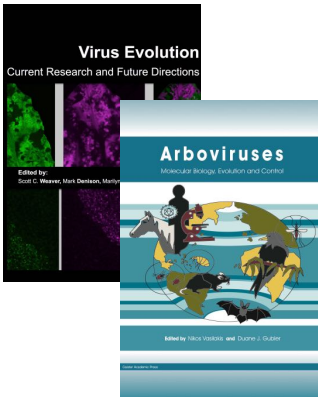

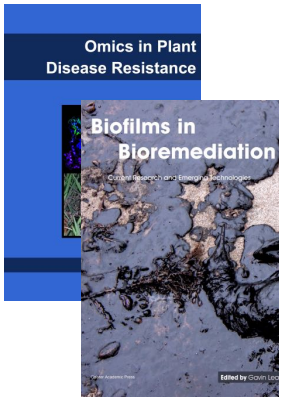

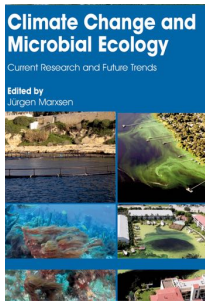

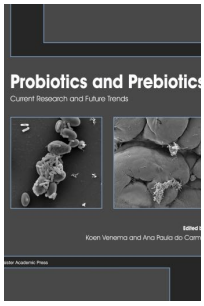

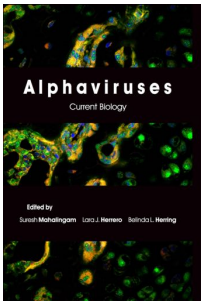

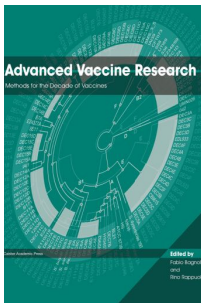

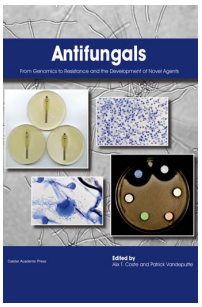

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function of heat shock proteins, the aims of this study were to determine the effect of chronic acid water feeding on heat shock proteins and intestinal barrier function.

Results

Mice fed chronic acid water develop an increase in HSP25 in the stomach, aorta, and kidney

To investigate the effect of the chronic acid water feeding on the local and systemic heat shock protein expression, HSP25, HSP72, and the constitutively expressed HSP73 levels were measured in intestinal segments from various regions of the intestinal tract as wells as in the liver, lung, aorta and kidney. There was no difference in HSP73 levels between acidified water fed versus tap water fed mice among these organs (data not shown). For HSP25 and HSP72, data were expressed as the ratio of HSP25 or HSP72 to HSP 73 to control for equal loading of protein. In intestinal segments, a statistically significant increase ($P<0.05$, Paired t-test, n=7) in HSP25, the predominant heat shock protein in the intestine, was increased in the stomach of mice fed acid water versus tap water (Fig. 1). No HSP25 was detectable in the jejunum, as we have previously reported (Wischmeyer *et al*., 2001). There was no difference in the expression of HSP72 in the stomach, jejunum and cecum between mice fed acidified and tap water (data not shown).

In the cecum, however, a statistically significant increase ($P<0.05$, Paired t test, n=7) in HSP25 expression was observed in mice drinking tap water compared to acid water (Fig. 2A). This unexpected result could be attributed to the finding that adherent bacteria, a major determinant

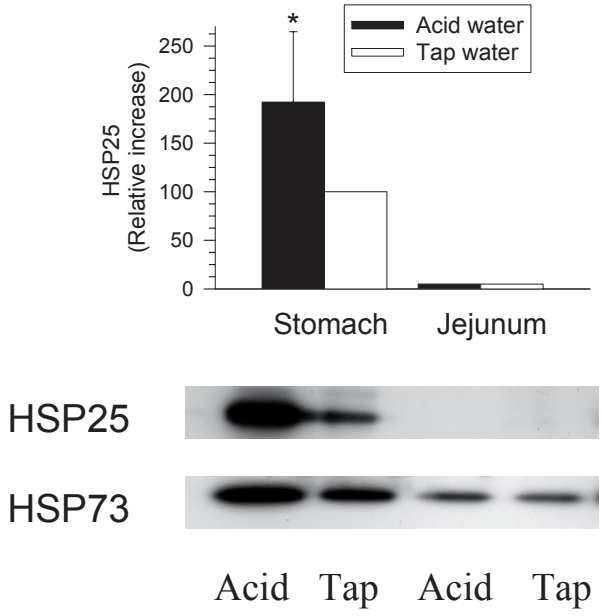

Fig. 1 Percentage increase in HSP protein expression in mice drinking acid water compared to tap water. HSP 25 and HSP73 protein levels were assessed by immunoblot analysis in mucosal samples of the stomach and jejunum in mice drinking either acid or tap water for 8 weeks. HSP values are expressed as relative changes between mice drinking tap water and acidified water using the ratio of HSP25 to the constitutively expressed HSP73. HSP25 expression was significantly increased (*: $P<0.05$, paired t test, n=7) approximately 2 fold in the stomach of mice drinking acid water. As previously reported, the jejunum did not express HSP 25.

of heat shock protein expression in intestinal mucosa, was increased in tap water fed mice and absent in acid water fed mice. As we have previously shown, the intestinal microflora directly induces the expression of heat shock proteins in the intestinal mucosa (Deitch *et al*., 1995; Kojima *et al*., 2003). Under normal conditions, the mouse cecum harbors the greatest quantity of bacteria adherent to the mucosa surface, the major aerobic species being *E. coli* (Rocha *et al*., 2001). In mice harboring normal flora, adherent *E. coli* is the most common bacteria shown to be directly responsible for the observed epithelial permeability defect induced by stress (Alverdy *et al*., 1999; Spitz *et al*., 1994). In the present study acid water feeding rendered the cecal mucosa sterile of gram negative bacteria (Fig. 2C), removing any possible influence of the intestinal microflora on HSP production. In order to account for the inducing effect of bacterial adherence on HSP25 levels between groups of mice, data were expressed as HSP25 per 1000 cfu of mucosally adherent bacteria. In this analysis the acid water treated mice displayed a higher level of expression of HSP25 compared to tap water treated mice (Fig. 2B). Since chronic acid water feeding in mice has been shown to have no effect on the pH of the cecum, we addressed whether acid water feeding might exert an indirect trophic effect on the intestinal mucosa by measuring the protein content in intestinal segments between mice. Interestingly, mice fed acid water had a significant increase ($P<0.05$, Paired t test, n=7) in mucosal protein content in the cecum compared to tap water fed mice (Fig. 2D).

In order to determine if a systemic effect of acid water feeding on HSP production could be demonstrated in this model, HSP expression was measured in systemic organs. HSP25 was undetectable in the liver tissues of mice and there was no difference in the levels of HSP25 in lung tissues. Levels of HSP72 expression in the liver and lung were not different between acid and tap water fed mice (data not shown). However, mice drinking acid water had a statistically significant increase ($P<0.05$, Paired t test, n=7) in HSP25 expression in the aorta and kidney compared to mice drinking tap water (Fig. 3A). Yet for HSP72, a statistically significant increase ($P<0.05$, Paired t test, n=7) in HSP72 was observed only in the aorta and not the kidney of mice fed acid water ($P<0.05$, Paired t test, n=7; Fig. 3B).

Chronic acid water feeding protects against lethal sepsis due to intestinal Pseudomonas aeruginosa*.*

In order to determine whether mice drinking acid water would be protected against infectious challenge by a relevant nosocomial pathogen with high lethal potential, we intracecally challenged mice with a clinical strain of *P. aeruginosa* following a 30% surgical hepatectomy. This model recapitulates a clinically relevant state of gut-derived sepsis. Mice fed acid water for 8 weeks were completely protected against gut-derived sepsis due to *P. aeruginosa* compared to mice drinking tap water (mortality rate at 48 hrs 5/7 versus 0/7, $P<0.05$ Fisher exact test; Fig. 4A). None of the acid water treated mice developed sepsis (ruffled fur, chromodacctyrrhea, diarrhea, etc.) and appeared completely healthy. Although bacterial cultures revealed a significant increase in the concentration of

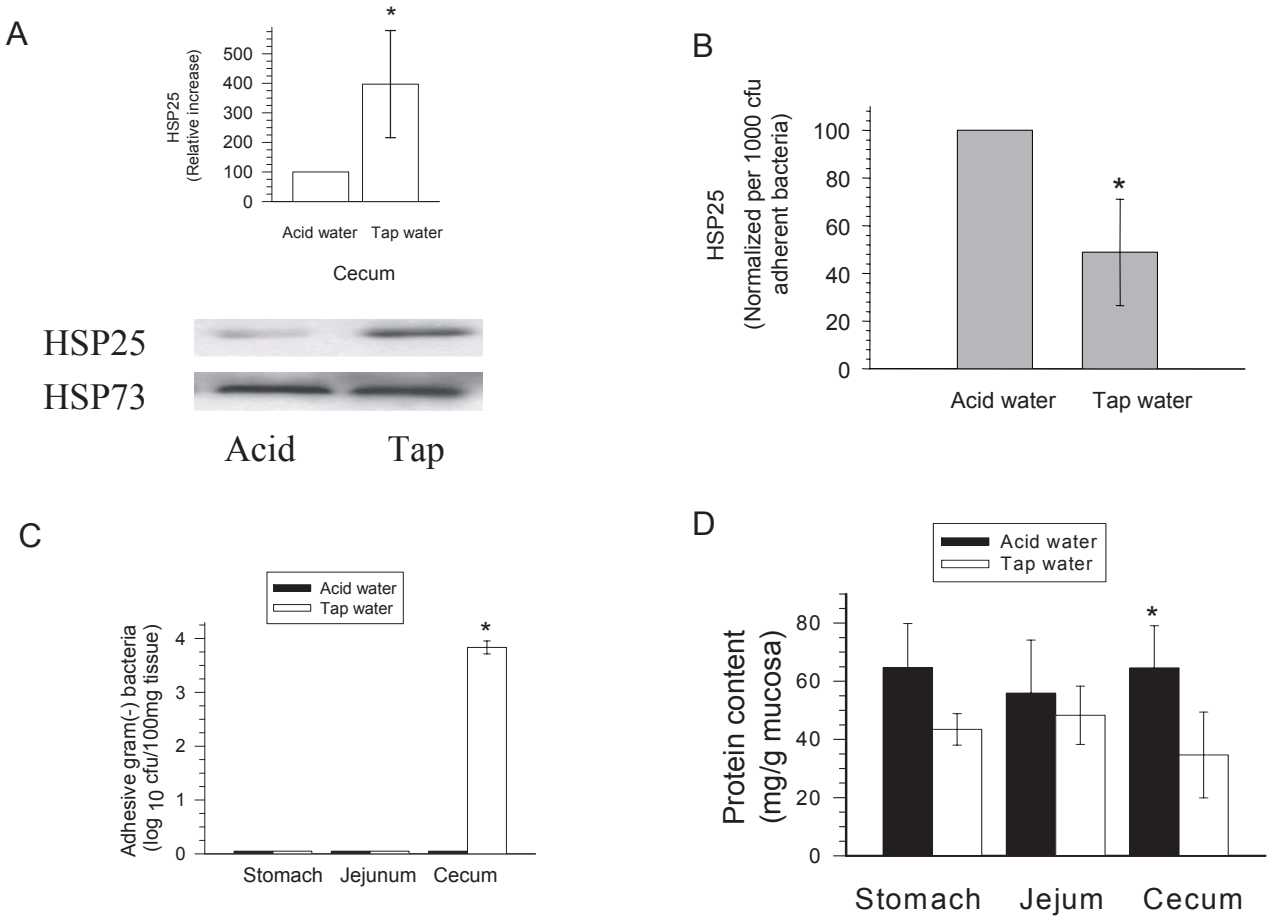

Fig. 2 The effect of acid water feeding on cecum protein content, adherence of gram negative bacteria to the intestinal mucosa, and cecal HSP25 expression. A. Although the relative values of HSP25 were increased in tap water fed mice compared to acid fed mice. B. when normalized for adherent bacteria the prime stimulus for HSP production in the intestinal mucosa, acid water fed mice displayed a relative increase in HSP25 production compared to their tap water fed cohorts (*: P<0.05, Paired t test, n=7). C. Quantitative culture of the intestinal mucosa for adherent gram-negative bacteria demonstrated that the stomach and jejunum were sterile in both acid and tap water fed mice. In the cecal mucosa, tap water fed mice had significant bacterial adherence to the mucosa whereas mice fed acid water had no bacterial adherence (*: P<0.01, Paired t test n=7). D. A statistically significant increase (*: P<0.05, paired t test, n=7) in protein content was observed in the cecal mucosa of mice drinking acid water.

P. aeruginosa in the liver and blood of mice drinking tap water versus acid water (P<0.05, Mann-Whitney test, n=7; Fig. 4B), there was no difference in the fecal or cecal mucosal concentration of *P. aeruginosa* between groups (P=NS, Mann-Whitney test, n=7; Fig. 4B).

Exposure of Caco-2 cells to an acid environment enhances their barrier function and protects against the barrier dysregulating effects of P. aeruginosa.

In order to expose Caco-2 cells to an acid environment, cells were grown overnight in 12% CO_2 or 5% CO_2 incubator. Under these conditions, the pH of the control media was 7.7 whereas cells grown at 12% CO_2 incubator developed a pH of 7.1. Cells grown under 12% CO_2 developed a statistically significant increase in TER (345 $\pm$ 31 versus 264 $\pm$ 20, P<0.05, Paired t test, N=6; Fig. 5A) compared to cells incubated at 5% CO_2. To determine if Caco-2 cells grown under acid conditions could resist the barrier dysregulating effect of *P. aeruginosa*, Caco-2 cells were apically inoculated with 10^7 cfu/ml of PA27853. TER was measured over 8 hours. Results summarized in Fig. 5B demonstrate that Caco-2 cells exposed to an acidic environment resisted the barrier dysregulating effects of *P. aeruginosa* (P<0.05, Paired t test, N=6). Quantitative cultures of the cellular media reveled that the bacterial counts were equal in both groups (7.84 $\pm$ 0.24 and 7.85 $\pm$ 0.23 cfu/ml P=NS, n=6).

Discussion

The intestinal tract reservoir is the anatomic site upon which severe physiologic stress exerts its most profound effect on organ function while at the same time it is the site in which the greatest microbial burden is accumulated (Pastores *et al*., 1996). The intestinal pathogens acquired during the course of critical illness might play a major role in the inflammation and mortality due to gut derived sepsis (Esposito and Noviello, 1997). Accumulating lines of evidence strongly point to the intestinal microflora as a major agent in driving and sustaining the systemic inflammatory response during severe catabolic stress (Kropec *et al*., 1993; Murono *et al*., 2003). Routine surveillance cultures of the intestinal tract microflora in

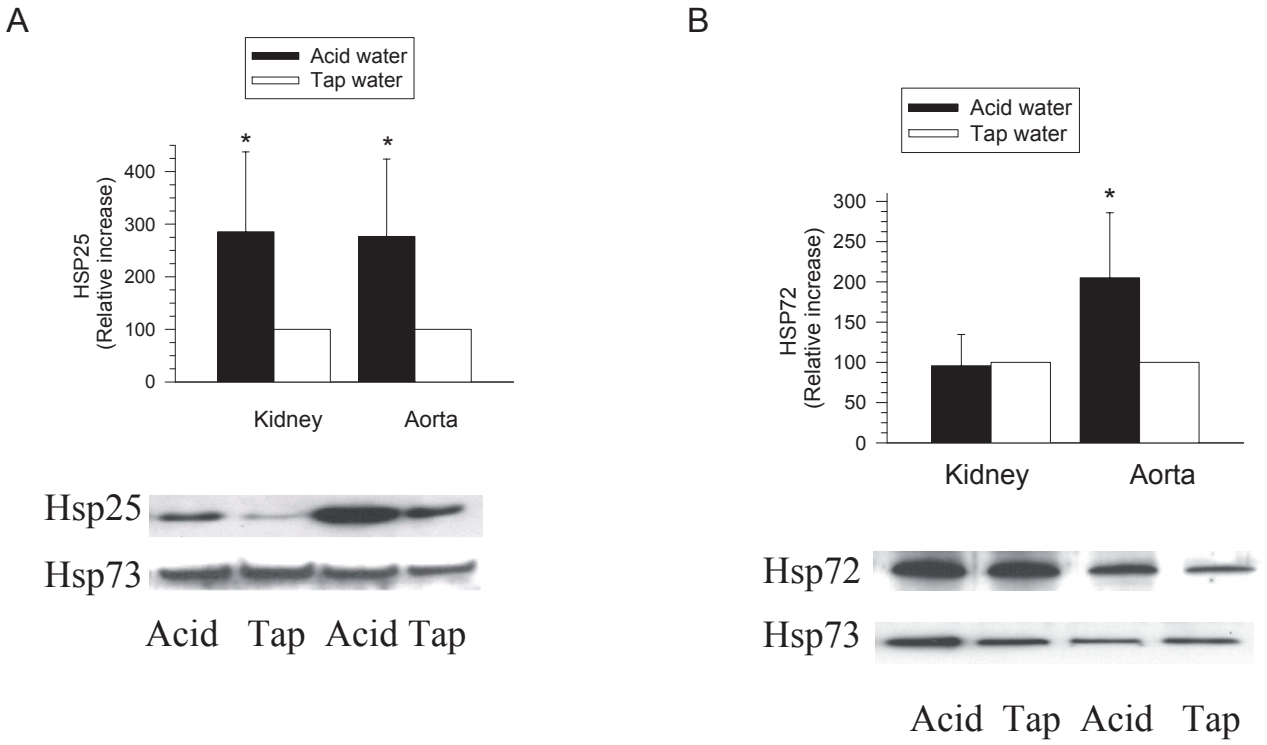

Fig. 3 Heat shock protein expression in the mouse aorta and kidney following acid water fed versus tap water fed. A. HSP25 expression measured by immunoblot was increased in the kidney and aorta of the mice drinking acidified water (*: P<0.05, Paired t test, n=7). B. HSP72 expression was increased in the aorta but not kidney of mice drinking acidified water (*: P<0.05, Paired t test, n=7).

intensive care unit patients suggest the normal flora are replaced by potent and virulent nosocomial pathogens such as *Streptococcus fecalis, Candida albicans*, and *Pseudomonas aeruginosa*.

Our laboratory has focused on the pathogenesis of intestinal *P. aeruginosa* since its mere presence in the intestinal tract of a critically ill patient is associated with a 4-fold increase in mortality, independent of its dissemination to remote organs (Marshall *et al*., 1993). Molecular analysis of blood and fecal isolates of *P. aeruginosa* from critically ill septic patients has established that the intestinal tract is the single most important reservoir for subsequent infection with this pathogen (Arbo *et al*., 1998). A recent surveillance study of 35,790 non-duplicate, gram-negative aerobic bacteria isolated from intensive care units (ICUs) in 43 US states identified *P. aeruginosa* as the most common gram-negative species isolated among critically ill patients (Neuhauser *et al*., 2003). *P. aeruginosa* carries the highest case fatality rate of all nosocomial pathogens and pre-emptive decontamination of the feces of intensive care unit patients of this pathogen is associated with a increase in survival (de Jonge *et al*., 2003).

There have been numerous clinical and experimental attempts to manipulate the gut contents during surgical and physiologic stress in an effort to reduce systemic inflammation and organ dysfunction (Rotstein, 2000). Perhaps the most widely prescribed regimen has been used in patients with hepatic insufficiency was lactulose and neomycin to manipulate the composition and numbers of intestinal bacteria to reduce hepatic encephalopathy (Weber, 1996). Although there have been numerous trials that have demonstrated a significant benefit of selective antibiotic decontamination of the intestinal tract in preventing sepsis and mortality in critically ill patients, there is continued reluctance for this approach due to the fear that it will lead to the emergence of resistant pathogens.

Manipulation of gut contents by acidification could be a more rational and less problematic approach to attenuate the pro-inflammatory potential of the intestinal microflora during critical illness. This practice was originally designed in response to several case reports in which enteral formulas were found to be contaminated by highly virulent pathogens. Subsequently numerous animal studies have established that chronic acid water feeding reduces the gram-negative count within the intestinal tract as far distally as in the cecum (Mehall *et al*., 2001). In animal models of necrotizing enterocolitis, acid feeding has been reported to be highly preventative (Carrion and Egan, 1990). The presumptive mechanism of this effect has been explained to be a direct effect of the acid environment on bacterial growth patterns. Yet it seems somewhat implausible that acidified water feedings would retain their topical effect through the entire course of the intestinal tract given that orally administered acid should be diluted and buffered by pancreaticobiliary and enteral secretions. In fact, the cecal pH measured during acid formula feeding has been reported to be normal (Chaveerach *et al*., 2004; Mehall *et al*., 2001). In addition a significant volume of water or feedings are absorbed within the upper third of the small bowel and therefore an oral acid load ought not to reach or directly affect the intestinal pH at such a remote site. Finally, the pH in the colon is highly dependent on the composition and metabolism of intestinal bacteria primarily due to the production of CO_2 and the production of volatile short chain fatty acids by anaerobic bacteria.

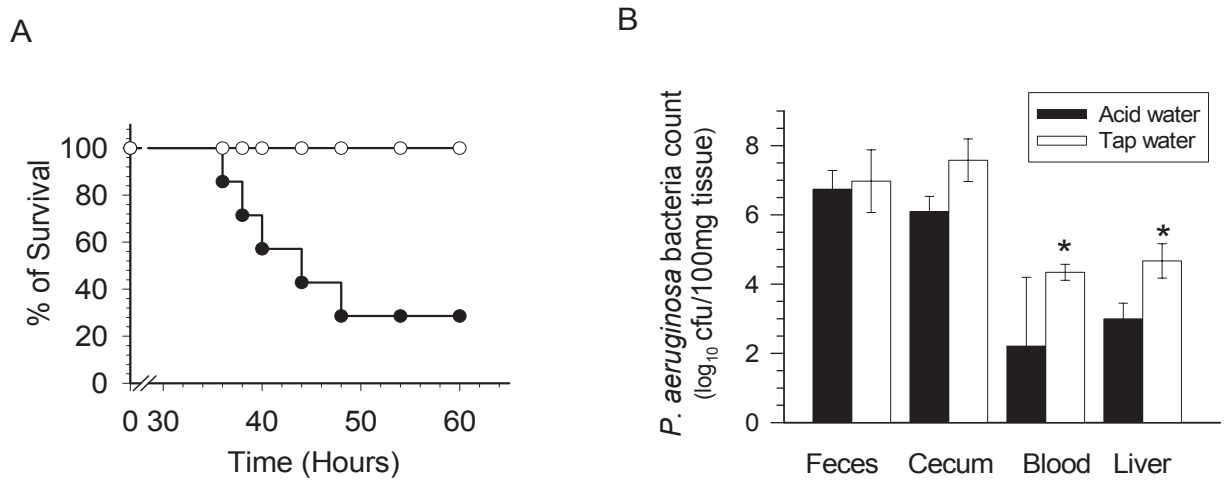

Fig. 4 Acid water feeding protects mice from lethal gut derived sepsis and is associated with a decrease in *P. aeruginosa* bacterial counts in the blood and liver. A. Survival curve demonstrated that mice fed acid water had lower mortality (0/7) following hepatectomy and cecal instillation of *P. aeruginosa* compared to mice fed tap water (5/7). B. Selective quantitative culture of feces, cecal mucosa, blood, and liver for *P. aeruginosa* demonstrated that chronic acid feeding significantly decreased the recovery of *P. aeruginosa* in the blood and liver (*: P<0.05, Mann-Whitney test, n=7).

These factors do not appear to be affected in animal fed acidified water (Chaveerach *et al*., 2004).

In this present study we examined the effect of acidified water on the quantity of gram negative bacteria adherent to the cecal mucosa, a reliable measure of the balance between the luminal microbial load and local mucosal defense. We used media selective for gram-negative aerobic bacteria since they are the most common bacteria to adhere to the mucosal epithelium. The cecum harbors the highest bacterial count in the mammalian intestine, and under normal circumstances, bacteria adhere to the cecal mucosa at concentrations of less than 10^5 cfu/g tissue (Hendrickson *et al*., 1999). We have previously shown that during stress, gram-negative bacteria adhere to the cecal mucosa at concentrations of greater than 10^5 cfu/g tissue and induce a significant defect in tight junctional permeability (Hendrickson *et al*., 1999). In the present study, acid feeding resulted in an essentially sterile cecal mucosa and a significant increase in mucosal protein content. Since acid water feeding has been previously shown to increase intestinal glutamine uptake, enhancement of local immune elements could have played a role in this observation. The finding that mucosal protein concentration was increased in acid fed mice is of interest, and could reflect a general increase in amino acid uptake as a result of chronic acid exposure and a positive trophic effect on the mucosal epithelium. Further work will be necessary to clarify and extend these preliminary findings.

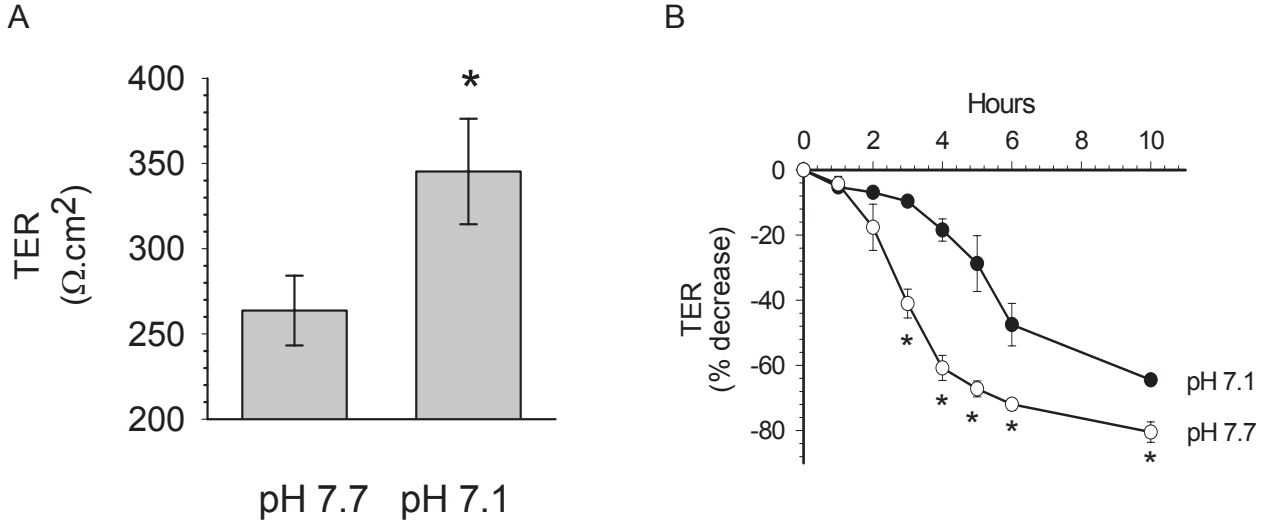

Fig. 5 Cultured intestinal epithelial cells exposed to an acid environment develop enhanced barrier function to *P. aeruginosa*. A. Exposure the intestinal epithelial cell line Caco-2 cell to 12% CO_2 resulted in a pH of 7.1 in the culture media and significantly increased the TER following overnight exposure. (N=6, *: P<0.05, Paired t test). B. An acid environment significantly attenuated the decrease in TER in Caco-2 cells apically inoculated with 10^7 cfu/ml of *P. aeruginosa* (•= acid exposure, o = control, * P<0.05, Paired t test, n=6). There was no difference in the final concentration of *P. aeruginosa* in the media between acid exposed Caco-2 cells and control cells (7.84 ± 0.24 versus 7.85 ± 0.23 cfu/ml, P=NS, n=6).

Acid water feeding also resulted in a significant increase in heat shock protein 25 concentration in the stomach, the site in which the acid exposure was most direct. The pH of the mouse stomach has been estimated to be approximately 4, whereas the acidified water in the present study had a pH 2.6. In the cecum however, HSP25 was increased in tap water fed mice. Previous work from our laboratory has demonstrated that among the various factors that can affect HSP expression in the mammalian intestine, exposure to intestinal bacteria appears to play a major and predominant role (Deitch *et al*., 1995; Kojima *et al*., 2003). Animal models in which bacterial overgrowth in intestinal loops are created demonstrate the highest induction of intestinal HSP production (Arvans *et al*., 2005). Conversely, mice decontaminated of their intestinal flora demonstrate a significant reduction in intestinal HSP concentration.

In an attempt to control for the effect of intestinal bacterial induction of heat shock protein production, we expressed HSP25 concentration as a ratio of HSP25/1000 cfu of adherent bacteria. Using this calculation, HSP25 concentration was actually increased in mice fed acid water compared to tap water fed mice, perhaps demonstrating the ability of the mucosa in acid fed mice to express a significant amount of heat shock protein despite the lack of any inducing bacteria. However because both aerobic and anaerobic bacteria are capable of inducing heat shock proteins in the intestinal mucosa, further work will be necessary to clarify the direct versus indirect effects of acid water feeding on cecal mucosal HSP production.

The most striking finding in this study was the increase of HSP25 expression in the aorta and kidney of mice on chronic acid water feeding. It is well established that chronic acid water feeding in mice can affect the metabolism of systemic tissues in a variety of sites. Under normal conditions, an acid load presented to the systemic circulation is readily buffered by intracellular and extracellular systems. In mice, chronic acid water feeding has been shown to result in a compensatory mild acidosis at day 7 ranging from pH 7.34–7.36 (Heyland *et al*., 1999). The very systems that might regulate these responses could in turn activate HSP production. The finding in the present study that HSP25 is increased in systemic tissues such as the aorta and kidney suggests that, analogous to ischemic pre-conditioning, chronic acid water feeding could condition various tissues to express stress proteins. Heat shock protein expression in both epithelial and endothelial cells has been shown to confer significant protection against a variety of stressors including bacterial invasion (Kojima *et al*., 2003), alteration in permeability (Chen *et al*., 2001; Venkatraman *et al*., 2003), ischemia reperfusion injury (McCormick *et al*., 2003), and cytokine release (Ropeleski *et al*., 2003). In most of these models, heat shock protein expression is induced by exposing tissues or whole animals to conditions of hyperthermia (Chen *et al*., 2001; McCormick *et al*., 2003). Interestingly, exposure of cells, tissues, or whole animals to hyperthermia itself leads to significant acidosis (Marder *et al*., 1990). Data from the present study raise the possibility that hyperthermia may increase HSP production, in part, via alterations in cellular pH as a secondary phenomenon. To our knowledge, there are no previous reports demonstrating that acid exposure increases the production of heat shock proteins in cells or tissues.

In order to test the hypothesis that the intestinal mucosa of mice is more resistant to the effects of virulent nosocomial pathogens as a result of acid water feeding, we assessed the ability of the cecal mucosa to contain the lethal effects of *P. aeruginosa* following direct cecal introduction in mice subjected to surgical hepatectomy. We have previously shown that instillation of *P. aeruginosa* into the cecum of mice subjected to a 30% hepatectomy results in lethal gut-derived sepsis (Alverdy *et al*., 2000). The mechanism by which *P. aeruginosa* induces lethality in this model is by inducing a defect in intestinal barrier function to major cytotoxins of this organism such as exotoxin A and elastase. In the present study, acid water feeding completely protected mice against lethal sepsis due to cecal *P. aeruginosa*. No differences in the luminal or cecal mucosal bacterial counts of *P. aeruginosa* were detected between acid and tap water fed mice, suggesting that the protective effect of acid water feeding was not due to its direct effect on bacterial counts within the cecum. Following *P. aeruginosa* instillation into the cecum, both groups of mice were given tap water only in order to avoid the possible direct effects of acid water on the cecal *P. aeruginosa* bacterial population. Therefore it appeared that chronic acid water administration to mice was not directly bactericidal against *P. aeruginosa* in this model, suggesting that alternative mechanisms are responsible for its observed protective effect.

A significant attenuation of bacterial translocation to the systemic circulation was observed in acid water fed mice which could be interpreted as evidence of enhanced intestinal barrier function in response to chronic acid water drinking. Alternatively, it is possible that the lower rates of recovery of bacteria in the liver and blood of mice drinking acid water could be due to enhanced systemic bactericidal activity as a result of acid preconditioning. We have previously shown in this model that the mortality following direct instillation of *P. aeruginosa* into the cecum occurs independent of bacterial translocation (dissemination) into the systemic compartment (Alverdy *et al*., 2000). In fact, intravenous or intraperitoneal instillation of an equal amount of *P. aeruginosa* results in no mortality in this model. The main mechanism by which *P. aeruginosa* induces mortality in this model is by creating a tight junctional permeability defect to exotoxin A or elastase, two lethal cytotoxic exoproducts of *P. aeruginosa*. When present in the systemic compartment, exotoxin A directly affects endothelial integrity causing severe fluid depletion from the intravascular space and shock(Wick *et al*., 1990). Since translocation of bacteria is primarily a transcellular event, and alterations in tight junctional permeability a paracellular event, acid preconditioning in the present study could have resulted in a greater enhancement of the latter property in the mucosal epithelium resulting in protection against mortality (Chen *et al*., 2001; Delarue *et al*., 1998). As a matter of speculation, acid water fed might have prevented *P. aeruginosa* toxin permeation across the epithelium resulting in protection against mortality with translocation acting as a surrogate marker of a generalized alteration in barrier function in this model.

In order to clarify this issue, we applied *P. aeruginosa* directly onto the human intestinal epithelial cell line, Caco-2. Acid exposure alone enhanced the barrier function of Caco-2 cells as assessed by an increase in cellular TER compared to cells grown at a more neutral pH. In addition, Caco-2 cells exposed to an acid environment resisted the barrier dysregulating effects of *P. aeruginosa* despite being exposed to a significant concentration of bacteria. Although the mechanism(s) remain to be clarified, an acid environment appears to have had a significant effect on the barrier function of the intestinal epithelium both *in vitro* and *in vivo.* Whether this observation is due to a higher concentration of glutamine within cells or up-regulation of local heat shock proteins will require further study.

Data from the present study have important implication both clinically and experimentally. Clinically, chronic acid water feeding could represent a viable strategy to precondition tissues in a manner analogous to ischemic preconditioning. Both ischemic preconditioning and hyperthermic preconditioning have been shown to improve survival in animals to a variety of subsequent insults including endotoxin or pathogen challenge, hypoxia, and tissue injury. Chronic acid water feeding could induce a compensatory acidosis that provides cells with a physiologic signal to mount a generalized cytoprotective response. If proved to be effective, this strategy will be feasible to apply in anticipation of a large stress such as prior to major surgical intervention. In terms of the use of chronic acid feeding as a routine practice to pathogen-specific free animals, the implications of the present study are profound. Virtually all animal care facilities that perform basic science research have adopted the policy of chronic acid water feeding to prevent intestinal colonization by *Pseudomonas aeruginosa*. This is especially prevalent in barrier facilities where specialized breeding takes place. The effect of such practice as a confounding variable in immune and physiologic studies should be considered.

Materials and methods

Bacterial strains, epithelial cell lines

In experiments involving *P. aeruginosa,* strain ATCC 27853 (PA27853), a non-mucoid clinical strain originally isolated from a blood culture was used. For experiments in which the *in vitro* effect of acid exposure on epithelial barrier function was determined, Caco-2 cells, a well-characterized human colon epithelial cell line that maintain a stable transepithelial electrical resistance (TER) (Turner *et al*., 1999), were used. Cells were grown confluently in transwells (Costar, Cambridge, MA) and TER monitored by specific electrodes (World Precision Instruments, Sarasota, FL) as described.

Mouse model of lethal sepsis due to intestinal P. aeruginosa

All experiments were approved by the Animal Care and Use Committee at the University of Chicago. Inbred Balb/c mice weighing 20g – 25g were used for all experiments. Mice were kept in individual wire bottom cage to avoid coprophagia during the entire experimental period**.** The mouse model of lethal sepsis due to intestinal *P. aeruginosa* involves direct instillation of *P. aeru*ginosa into the cecum following a 30% surgical hepatectomy. This model results in a mortality rate of 75–100% and has been described in detail previously (Alverdy *et al*., 2000). Briefly, animals are anesthetized (Ketamine 100mg/kg, xylazine10mg/kg, atropine 0.04mg/kg intraperitoneally). Through a midline incision, the floppy left lobe of liver is excised using electrocautery for a 30% resection. Specimens are weighed and the ratio of excised liver to body weight is calculated to confirm uniformity of the resection. Following the hepatectomy, a puncture into cecum with 27-g needle is made to inject live bacteria. 200μl (~ 10^8 cfu/ml) of *P. aeruginosa*, grown overnight in tryptic soy broth, is injected directly into the cecum. The cecal puncture site is tied off with a 4–0 silk suture and swabbed with iodine and the abdomen is closed. Animals are allowed water ad libitum only for the remainder of the study period. At the time of sacrifice, cecal contents, washed cecal mucosa, liver, and blood are harvested and quantitatively cultured on *Pseudomonas* isolation agar (PIA; Becton&Dickinson, Sparks, MD).

Assessment of bacterial adherence to the intestinal mucosa

In order to determine the effect of acid water feeding on the colonization resistance of the intestinal mucosa, we measured the quantity of gram-negative bacteria adherent to the mucosa in various regions of the intestinal tract. We have previously demonstrated that under normal circumstances, the washed proximal intestinal mucosa (stomach, jejunum) is essentially sterile, whereas in the distal mucosa (cecum, colon), gram-negative bacteria adhere to the epithelium at concentrations below 10^5 cfu/g, a quantity considered not to be associated with any alteration in intestinal mucosal physiology or pathology (Rocha *et al*., 2001). Previous studies in our laboratory have established that adherence of gram negative bacteria to the intestinal epithelium at concentrations of greater than 10^5 cfu/g, induces a significant defect in both epithelial tight junctional permeability and mucosal ion physiology (Hendrickson *et al*., 1999). Therefore this assay not only assesses the ability of the intestine to resist colonization by gram-negative bacteria, but also provides information on the relationship between the luminal microflora and the intestinal epithelium. Thus, segments of the stomach, jejunum, ileum, and cecum were vigorously washed in PBS by vortexing specimens, homogenizing them, and then quantitatively culturing them on MacConkey agar (Becton&Dickinson, Sparks, MD).

Quantitative bacterial counts in tissues

In experiments in which *P. aeruginosa* was instilled into the cecum of mice following 30% surgical hepatectomy, feces, washed cecal mucosa, liver, and blood, were similarly weighed, homogenized, and quantitatively cultured on PIA in order to determine both adherence to the intestinal mucosa and dissemination of *P. aeruginosa* following cecal inoculation as previously described. For *in vitro* experiments, quantitative culture of *P. aeruginosa* in the media of Caco-2 cells was performed as previously described in order determine the final concentration of live *P. aeruginosa* following apical inoculation (Wu *et al*., 2004).

Measurement of mucosal protein content and heat shock proteins

Heat shock protein analysis was carried out by harvesting organs (stomach, jejunum, cecum, liver, lung, aorta and kidney) and placing them directly into iced PBS. The stomach, jejunum and cecum were washed thoroughly in sterile PBS and the mucosa scraped and weighed. The scraped mucosa and organs were homogenized in lysis buffer (10 mM Tris pH 7.4, 5 mM $MgCl_2$, 50 U/ml DNAse, and RNAse, containing a complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). An aliquot was removed for measuring total protein concentration by micro BCA methods (Pierce, Rockford, IL) and the remainder was put into laemmli stop solution. The protein content per gram tissues was calculated. 10ug per sample was heated at 75° C for 10min. and separated by 10% SDS PAGE system. The separated proteins were immediately transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in TBST for 1 hour followed by incubating with the specific anti-HSP25 antibody (SPA 801; Stressgen, Victoria, BC, Canada), anti-HSP72 antibody (SPA810; Stressgen) or anti-HSP73 antibody (SPA 815; Stressgen) at 4°C overnight. After application of an HRP conjugated secondary antibody for 1hour, heat shock protein expression was detected by ECL reagent (Amersham, Piscataway, NJ). The density of HSP25, HSP72 and HSP73 were measured by NIH imager. The ratio between HSP25 or HSP72 and HSP73 was calculated. The relative changes of the heat shock protein expression were calculated by setting the lower expression group as 100%.

Assessment of Caco-2 barrier function

Caco-2 cells were grown on 0.3 cm^2 transwells (Costar, Cambridge, MA) for 10–14 days to reach confluence. In order to assess tight junction barrier function of the confluent monolayers, transepithelial electrical resistance (TER) was dynamically measured across Caco-2 cells using a EV-OHM resistance measurement apparatus (World Precision Instruments, Sarasota, FL) over 8 hours as previously described. Results were expressed as % change in TER.

Experimental protocol I: The effect of chronic acid water feeding on mucosal protein content, organ heat shock proteins expression, and bacterial adherence to the intestinal mucosa

In order to determine the effect of chronic acid water feeding on bacterial adherence to the intestinal mucosa and local and systemic heat shock protein concentration, mice were randomly assigned to two water feeding protocols. Group I (n=7) were allowed to drink tap water ad libitum and group II (n=7) acidified water adjusted to a pH of 2.6 with hydrochloric acid (HCl). Mice were fed the water regimens for 8 weeks and were allowed free access to their regular diet of mouse chow. Mice were weighed daily and at 8 weeks were sacrificed. At sacrifice, constant segments of the stomach, jejunum, ileum, and cecum were obtained for analysis of bacterial adherence, total protein content, and heat shock proteins. In addition, constant segments of the aorta, liver, lung, and kidney were obtained for heat shock protein analysis.

Experimental protocol II: The effect of chronic acid water feeding on mortality from gut-derived sepsis due to Pseudomonas aeruginosa

In order to assess the ability of chronic acid feeding to protect against lethal gut derived sepsis, mice were randomly assigned to receive tap water (n=7) or acidified water (n=7) ad libitum for 8 weeks. At 8 weeks mice were inoculated directly into cecum with 10^8 cfu/ml of live *P. aeruginosa* strain 27853 following a 30% surgical hepatectomy as outlined above. Both groups of mice were then fed tap water only for the next 48 hours and followed for the development of sepsis and mortality. All mice were sacrificed at the time of death (or when moribund) or at 48 hours and the stomach, jejunum, ileum, cecum, liver, and blood were harvested and quantitatively cultured for *P. aeruginosa* on PIA.

Experimental protocol III: The effects of acid exposure on the barrier function of cultured intestinal epithelial cells apically inoculated with Pseudomonas aeruginosa

To determine the direct effect of acid exposure on intestine epithelial cell barrier function, human intestinal epithelial cells (Caco-2) were exposed to normal pH conditions or an acid environment by incubating cells at either 5 or 12% CO_2 overnight. This procedure provided a stable pH throughout the experimental protocol and avoided the bacteriacidal effect of adding HCL. The initial TER of Caco-2 cells was compared between normal (5% CO_2) and acid environment (12% CO_2). The percent alteration in TER was dynamically assessed over time following apical inoculation of 10^7 cfu/ml of live *P. aeruginosa*, a pathogen previously demonstrated to induce a more than 50% decrease in the TER of Caco-2 cells with 4 hours. Cultures were monitored for changes in pH and bacterial counts during the course of the experiment.

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