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Heat Shock Response and Gastrointestinal Mucosal Defense

Doctoral dissertation

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ABSTRACT

Gastrointestinal mucosal integrity is maintained by both physiological and anatomical factors. In case of failure of this maintenance system either superficial injury or frank macroscopic ulceration occurs. The gastrointestinal mucosa responds to injury by activation of several intrinsic protective systems. With superficial mucosal injury, the mucosal integrity is restored by rapid epithelial restitution. Increased cell proliferation, apoptosis or synthesis of cytoprotective heat shock proteins, i.e. induction of the heat shock response, may occur in response to injury as well. Heat shock proteins repair damaged proteins, inhibit apoptosis, and protect against deep mucosal injury. In preconditioning, induction of heat shock proteins by a sublethal stimulus such as hyperthermia is used to protect the tissue from subsequent injury. Heat shock also results in the activation of eicosanoid pathways, which are closely related to mucosal protection.

In this study, the effect of heat shock preconditioning on restitution was studied in an Ussing chamber perfusion system with isolated guinea pig gastric mucosae in simulated physiologic conditions *in vitro*. Tissue response was modulated with agents interfering with protein synthesis and eicosanoid pathways. Analysis of transmucosal resistance, histology, expression of heat shock proteins, proliferative and apoptotic markers and activity of the major apoptotic effector enzyme caspase-3 was performed. As a model of deep transmucosal injury, the effect of intestinal ischemia and reperfusion *per se* on the heat shock response was studied in pigs *in vivo* and analysis of the heat shock response at various levels and timepoints was performed.

Heat shock preconditioning induced expression of heat shock proteins and transient alterations in transmucosal resistance via mechanisms dependent on both protein synthesis and eicosanoid pathways and without causing a morphological injury or activation of apoptotic cascades. Preconditioning also suppressed cell proliferation and inhibited restitution by a mechanism involving both protein synthesis and eicosanoid pathways. Heat shock preconditioning modulated caspase-3 activity via a mechanism dependent on eicosanoid pathways. During normothermia, caspase-3 activity in the gastric mucosa was regulated by a mechanism involving eicosanoid pathways and protein synthesis. Reperfusion induced a transcriptionally mediated heat shock response in the small intestine.

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Kuopio, May 2003

Niku Oksala

ABBREVIATIONS

AA	arachidonic acid
Ac-DEVD-CHO	N-acetyl-Asp-Glu-Val-Asp-Aldehyde
apO ₂	arterial blood partial tension of oxygen
apCO ₂	arterial blood partial tension of carbon dioxide
ATPase	adenosine triphosphatase
CGRP	calcitonin gene regulated peptide
CHX	cycloheximide
CO ₂	carbon dioxide
cyt c	cytochrome c
DMEM	Dulbecco's Modified Eagle Medium
dpCO ₂	intestinal mucosal-arterial gradient of partial tension of carbon dioxide
EGF	epidermal growth factor
FGF	fibroblast growth factor
HS	heat shock
HSE	heat shock element
HSF1	heat shock factor 1
Hsp	heat shock protein
IN	indomethacin
mpCO ₂	intestinal mucosal partial tension of carbon dioxide
mRNA	messenger ribonucleic acid
NO	nitric oxide
NSAID	nonsteroidal anti-inflammatory drug
NT	normothermia
PBS	phosphate buffered saline
PLA ₂	phospholipase A ₂
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error of mean
SMA	superior mesenteric artery
TGF-β	transforming growth factor beta
TNF	tumor necrosis factor
Q	quercetin

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the roman numerals.

- I Oksala NKJ, Paimela H, Alhava E (2002) Preconditioning hyperthermia inhibits restitution of isolated guinea pig gastric mucosa. *Eur Surg Res* 34: 339-345.
- II Oksala NKJ, Paimela H, Alhava E (2002) Heat-shock preconditioning affects restitution of isolated guinea pig gastric mucosa by an arachidonic acid and protein synthesis dependent mechanism. *Scand J Gastroenterol* 37:1366-1373.
- III Oksala NKJ, Oksala A, Paavonen T, Alhava E, Paimela H (2003) Heat shock preconditioning modulates proliferation and apoptosis after superficial injury in isolated guinea pig gastric mucosa via an eicosanoid and protein synthesis-dependent mechanism. *APMIS*, in press.
- IV Oksala NKJ, Alhava E, Paimela H. Heat shock preconditioning and eicosanoid pathways modulate caspase-3 like activity in superficially injured isolated guinea pig gastric mucosa. Submitted for publication.
- V Oksala NKJ, Kaarniranta K, Tenhunen JJ, Tiihonen R, Heino A, Sistonen L, Paimela H, Alhava E (2002) Reperfusion but not acute ischemia in pig small intestine induces transcriptionally mediated heat shock response in situ. *Eur Surg Res* 34:397-404.

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1. INTRODUCTION

The gastrointestinal mucosa is continuously exposed lumenally to aggressive physical and chemical factors, which may compromise mucosal integrity. These factors include both dietary factors and physiological luminal secretions. The integrity of the gastrointestinal mucosa is maintained by mucosal defense systems consisting of both anatomical and physiological components - a mucus layer, epithelial cells, intercellular junctions, cellular buffering systems, mucosal microcirculation, rapid epithelial repair by cell migration, i.e. restitution, or repair mechanism analogous to wound healing accompanied by an inflammatory reaction. Depending on the degree of insult, the balance between defense mechanisms and aggressors may become perturbed, resulting in varying degrees of mucosal injury.

Heat shock preconditioning is a process, in which tissue is exposed to sublethal stress to increase its tolerance against subsequent potentially lethal stress. This process has been subjected to extensive research. During the last decade, it has become apparent that it is mediated at least partially by synthesis of a class of molecular chaperones, heat shock proteins. Heat shock proteins assist in re-folding of denatured proteins and inhibit stress-induced apoptosis. In addition to changes in protein synthesis, there are complex alterations in the eicosanoid pathways, including metabolism of arachidonic acid to leukotrienes and prostaglandins, which contribute largely to the gastric mucosal defense. The ability of the heat shock response to protect the tissue varies depending on the type of injury and the types of cell and tissue.

To date, all the studies elucidating the significance of heat shock preconditioning in gastric diseases have been done using deep models of gastric ulceration, a mode of injury entirely different from a single superficial injury. Heat shock proteins have been proposed to play a role in protection against gastric ulceration. In the small intestine heat shock proteins have been shown to protect against injury induced by ischemia and reperfusion. Although there are data on the cytoprotective role of heat shock proteins in intestinal ischemia-reperfusion, the effects of ischemia and reperfusion *per se* on the small intestinal heat shock response have been poorly characterized.

This study was primarily focused on the effects of heat shock preconditioning on isolated gastric mucosa after superficial injury and on the effects of ischemia and reperfusion on the heat shock response in the small intestine.

2. REVIEW OF THE LITERATURE

2.1 Classification of mucosal injury

Gastrointestinal mucosa is offended by various endogenous and exogenous stressors during digestion. These include hydrochloric acid and pepsin, bile salts, shear stress and various forms of mechanical trauma during digestion and hyper- and hypo-osmolarity of the luminal contents (Allen et al., 1993). The mucosal injury ranges from apical plasma membrane wounding to frank macroscopic ulceration penetrating the whole mucosa.

In superficial (i.e. microscopical) injury, the continuity of the epithelium is impaired, as manifested by local denudation of the basement membrane. However, the basement membrane remains largely intact in this form of injury. Interestingly, intrinsic defense mechanisms are capable of reconstituting the epithelial integrity by cell migration, thereby preventing deeper injury. A clinical example of this type of recovery is the healing of mucosa after an endoscopic biopsy. A special entity of microscopic injury is plasma membrane wounding, in which a limited injury to the apical plasma membrane of a cell is followed by spontaneous resealing of the wound without cell death (McNeil and Ito, 1989). This type of injury is supposed to occur under normal, physiologic conditions in the gastrointestinal epithelium and to participate in the maintenance of epithelial integrity. This mechanism of wounding has also been suggested to act as a previously unrecognised and diffusion-mediated route for molecular traffic across the plasma membrane (McNeil and Ito, 1990). In experimental biology, the ability of the plasma membrane to spontaneously reseal without cell death enables the use of microelectrode puncture techniques (Kivilaakso and Kiviluoto, 1988).

A completely different entity is deep mucosal injury, as manifested by frank ulcerations extending through basement membrane all the way to the submucosa and accompanied by an inflammatory reaction. In this form of injury, there is migration of inflammatory cells and formation of fibrin-rich scar tissue, angiogenesis and tissue remodeling totally absent in the superficial form of injury. The time scale of healing of chronic ulcers is also different, they heal over a period of weeks or months by a process analogous to the healing of skin wounds (Silen and Ito, 1985, Silen, 1987).

Intestinal ischemia appears both as a chronic condition and as an emergency situation in mesenteric embolism or with surgery for aortic aneurysm. Ischemia results in disturbed integrity of both the mucosal barrier and of capillary endothelium, allowing translocation of luminal microbes and polymorphonuclear leucocytes into subepithelial

capillaries, respectively. Subsequent reperfusion flushes these agents into the systemic circulation, ultimately leading to the induction of an inflammatory cascade, with an overwhelming multiple organ dysfunction as the end result (Haglund, 1993, Haglund, 1994, Landow and Andersen, 1994, Tamion et al., 1997).

2.2 Experimental models of mucosal injury

In restitution research, the focus of interest is the pathophysiology of the surface epithelial cell layer. Therefore, the models applied are as specific as possible. The integrity of the epithelial cell layer is monitored either electrophysiologically in an Ussing chamber or by photography in cell-culture models. Primarily the Ussing chamber model was designed for ion-transport studies, but was later applied to restitution research (Ussing, 1950, Svanes et al., 1982, Rutten and Ito, 1983).

Of the various mechanical and chemical forms of inducing a superficial injury, the one utilizing hyperosmolar sodium chloride followed by isotonic fluid is physiological and the most widely documented (Silen, 1987, Silen, 1988). Hyperosmolar sodium chloride induces acutely cellular shrinkage and flow of isotonic fluid from the serosal side to the luminal side to balance the gradient. After a 5 min perfusion with hyperosmolar luminal solution, the fluid is changed to an isotonic one leading to acute cellular swelling and ultimately bursting of the most superficial cells. The flow of the isotonic serosal solution is the most important factor limiting the depth of the injury. The clearest benefit of this experimental model of superficial injury is that it can be reproduced easily and gives constant recovery (Silen, 1987, Silen, 1988). Some other models utilize ethanol, nonsteroidal anti-inflammatory drugs, acetic acid, boiled water, bile acids, and other surface-active detergents, especially Triton X-100. The problem with these models is that they are able to induce deep injuries also. Therefore, an injury limited to the most superficial part of the mucosa and leaving the gastric pits intact is very hard to reproduce. Luminal acetylsalicylic acid (Hingson and Ito, 1971), bile salts, and ethanol (Eastwood, 1985) cause extensive damage to the epithelium itself, but leave the tight junctions intact. These agents increase the conductance of the apical cell membrane to hydrogen ions backdiffusing from gastric lumen, leading eventually to intracellular acidosis and disruption of the epithelial cell layer (Allen et al., 1993).

2.3 Mucosal defense mechanisms

The gastric mucosa is defended against luminal aggressors by the following principal mechanisms: the secretion of mucus and bicarbonate, the properties of the apical plasma membrane, such as impermeability, gastric tight junctions, strictly regulated microcirculation, regulation of intracellular pH, the rapid epithelial restitution and proliferation and the endogenous protein synthesis dependent mechanisms including heat shock proteins (Figure 1).

2.3.1 Mucus

The mucus forms a continuous, firmly adherent layer adjacent the epithelium (Figure 1). In the gastric mucosa, presecreted mucus is stored in the intracellular vesicles of the epithelial cells. The mucus comprises two layers, a stable unstirred layer right next to the apical membranes of the epithelial cells and a more luminal, soluble layer mixed with the luminal contents (Allen et al., 1993). These two layers provide both a physical and a chemical barrier. The mucus layer possesses microscopical canals to allow the secretions, acid and pepsin to go through (Johansson et al., 2000). These channels are probably created by the high intraglandular pressure pushing acid and glandular mucus into the gel (Johansson et al., 2000). The more luminal loosely adherent mucus layer contributes to maintaining juxtamucosal pH at very low pH values if acid secretion and prostaglandin synthesis are inhibited (Phillipson et al., 2002). The mucus is rich in mucin glycoproteins, proteins, lipids and nucleic acids from exfoliated epithelial cells (Allen et al., 1993). The mucins account for the viscoelastic properties. The mucins are capable of binding water and bicarbonate also secreted from the epithelium, thereby forming a neutralizing functional layer blocking the backdiffusing hydrogen ions from luminal acidic secretions. The importance of prostaglandins in gastric mucosal protection is emphasized by the fact that the secretion of mucus is dependent on prostaglandin E₂ (Tani et al., 2002).

2.3.2 Apical plasma membrane impermeability, tight junctions and secretion of bicarbonate

The apical plasma membranes make it very hard for a hydrogen ion to diffuse back from the lumen to the intracellular space. Therefore, gastric epithelial cells are specialized to maintain a high pH gradient between the gastric lumen and the apical plasma membrane (Allen et al., 1993). It is disturbed by barrier breakers (Hills et al.,

1983, Goddard et al., 1990, Hills, 1996). It has been proposed that in isolated mucosae of the Necturi exogenous phospholipids account for the protection against acid and barrier-breakers by increasing this hydrophobicity (Kiviluoto et al., 1991). In addition to these properties of the plasma membrane, the well-defined tight junctions create an anatomical barrier against luminal acidity (Figure 1).

The transmembrane sodium gradient maintained by sodium-potassium-ATPase drives sodium-bicarbonate transport across the serosal membrane. Bicarbonate is transported transcellularly from the serosal side of the mucosa to the luminal side (Kiviluoto et al., 1993). In the apical cell membrane, a chloride-bicarbonate exchanger is operative and makes it possible for bicarbonate to enter the lumen. The tight junctions, especially in the fundic area, also allow low rates of passive diffusion via the paracellular pathways (Spenny et al., 1975) (Figure 1). The presence of acid in the lumen stimulates secretion of mucosal bicarbonate from the surface epithelial cells thereby serving as a self-activating defense mechanism. The secreted bicarbonate alkalinizes the mucus gel and replenishes the bicarbonate lost in the neutralization of luminal acid (Allen et al., 1993). Of importance, acid-secreting cells produce a significant amount of bicarbonate, which is transported via the mucosal microvasculature to the more superficial mucous cells to be secreted in to the lumen (Synnerstad et al., 2001) (Figure 1). Therefore, the acid-secreting mucosa is less vulnerable to luminal acid than the non-secreting mucosa (Synnerstad et al., 2001). The secretion of bicarbonate is controlled by prostaglandins (Takeuchi et al., 1999). In case of mucosal damage, a passive diffusion of interstitial fluid containing bicarbonate ions occurs through the mucosa towards the luminal space. This results in alkalization of the mucoid cap already formed over the damaged mucosa to allow undisturbed repair of the mucosa to continue (Wallace and McKnight, 1990).

2.3.3 Sodium-hydrogen transport and pH and volume regulation

In case back-diffusing hydrogen ions penetrate the apical plasma membrane, a specialized ion-exchange pump is activated, namely the sodium-hydrogen-exchanger. It extrudes the intruding hydrogen ions against their electrochemical gradient using the electrochemical gradient for sodium ions (Roos and Boron, 1981) and contributes therefore to the maintenance of intracellular pH upon luminal acidification (Kivilaakso and Kiviluoto, 1988) (Figure 1). It seems to be the main proton-extruding mechanism in isolated gastric glands or surface, chief, and oxyntic cells (Olender et al., 1984, Machen

and Paradiso, 1987). The net result of this phenomenon is alkalinization of the intracellular space. In addition to regulation of pH, this exchanger participates in the volume regulation by regulating intracellular sodium concentration. While hyperosmolarity and hypo-osmolarity are common occurrences in the gastric lumen, the volume regulation is needed to balance the epithelium daily (Carter et al., 1993). The sodium-hydrogen-exchanger contributes critically to repair of injury, since the effects of growth factors are mediated at least partially by stimulation of basolateral sodium-hydrogen-exchangers (Joutsu et al., 1996, Yanaka et al., 2002).

2.3.4 Capillary circulation

When hydrogen ions diffuse into the mucosa they must be rapidly eliminated to prevent tissue acidosis. Mucosal blood flow plays the central role in this process. The mucosal microcirculation accounts for effective neutralization of the invading hydrogen ions. The capillaries in the gastric mucosa are fenestrated, which facilitates transportation of bicarbonate formed in the parietal cells in the process of acid secretion to the surface epithelium via the bloodstream (Gannon et al., 1982, Gannon et al., 1984) (Figure 1).

2.3.5 Epithelial proliferation

Characteristic of the gastric epithelium is continuous proliferation, which maintains a continuous epithelial cell barrier (Silen and Ito, 1985, Eastwood, 1991, Allen et al., 1993). The cell cycle is divided into mitosis (M), which is followed by a silent gap (G1) and eventually by a phase characterized by synthesis of DNA (S). Subsequently, this is followed by a second gap (G2) during which the mitotic spindles are assembled. After mitosis, some cells may enter a special phase (G0), during which there is no DNA synthesis or mitosis. The half-life of surface epithelial cells is 1-2 days. The proliferative cells are located in the isthmus of glands. The majority of new cells migrate towards the surface while a fraction of cells migrates towards the bottom of the glands. The surface epithelial cells are completely replaced every few days. Renewal of the population of glandular epithelial cells requires several months (Willems, 1972).

2.3.6 Epithelial restitution

In case of superficial injury, which is limited to the basal lamina, the defect is repaired by a process of restitution characterized by rapid migration of remaining viable cells at a

speed of up to 2 $\mu\text{m}/\text{min}$ (Ito et al., 1984, Silen and Ito, 1985, Ito and Lacy, 1985, Lacy, 1987, Lacy et al., 1993, Lacy, 1995). Morphological studies have revealed that superficial microscopic damage always accompanies mucosal barrier disruption (Lacy, 1988, Silen, 1988). Immediately after the injury, the superficial epithelial cells exfoliate as a sheet from the basal lamina leaving gaps in the epithelial lining. The remnants of exfoliated cells and exudates form a mucoid cap (Wallace and McKnight, 1990). Mucoid cap ensures a favourable microenvironment for restitution to occur since an alkaline exudate is trapped beneath it (Wallace and McKnight, 1990). After a few minutes, the viable mucous neck cells undergo a shape change, becoming squamous and begin to extend lamellipodia to restore the integrity over the basal lamina. If the basement membrane is damaged or not present at all, then restitution is impaired (Lacy, 1987). As the lamellipodia from migrating cells make contact tight junctions are formed. Eventually, these cells transform back to cuboidal and ultimately columnar shapes. This morphological restitution is paralleled by restoration of electrical integrity and normal ion transport properties (Svanes et al., 1982, Rutten and Ito, 1983, Svanes et al., 1983). Complete restitution is achieved in as little as 15 min in the rat stomach *in vivo* and as long as 4 h in amphibian gastric mucosa mounted in an Ussing chamber (Svanes et al., 1982, Svanes et al., 1983, Ito et al., 1984). Requirements for restitution are nearly neutral luminal pH and the presence of Ca^{2+} ions (Svanes et al., 1982, Rutten and Ito, 1983, Critchlow et al., 1985). The process is controlled both by several humoral (growth factors, cytokines, trefoil peptides, adrenomedullin, NO, CGRP) and by structural (cytoskeleton, integrins, basal lamina) factors (Critchlow et al., 1985, Dignass and Podolsky, 1993, Paimela et al., 1993, Dignass et al., 1994, Miller et al., 1995, Yanaka et al., 1995, Yanaka et al., 1996, Riegler et al., 1996, Fukuda et al., 1999, Poulsen et al., 1999), which also control the rate of epithelial proliferation. In addition, prostaglandin E_2 , a product of arachidonic acid, is involved in the process through production of mucus and bicarbonate to maintain an optimal micromilieu for restitution (Takeuchi et al., 1999, Takahashi et al., 1999) and to maintain and re-establish the mucosal barrier (Bliklager et al., 1997b, Bliklager et al., 2001).

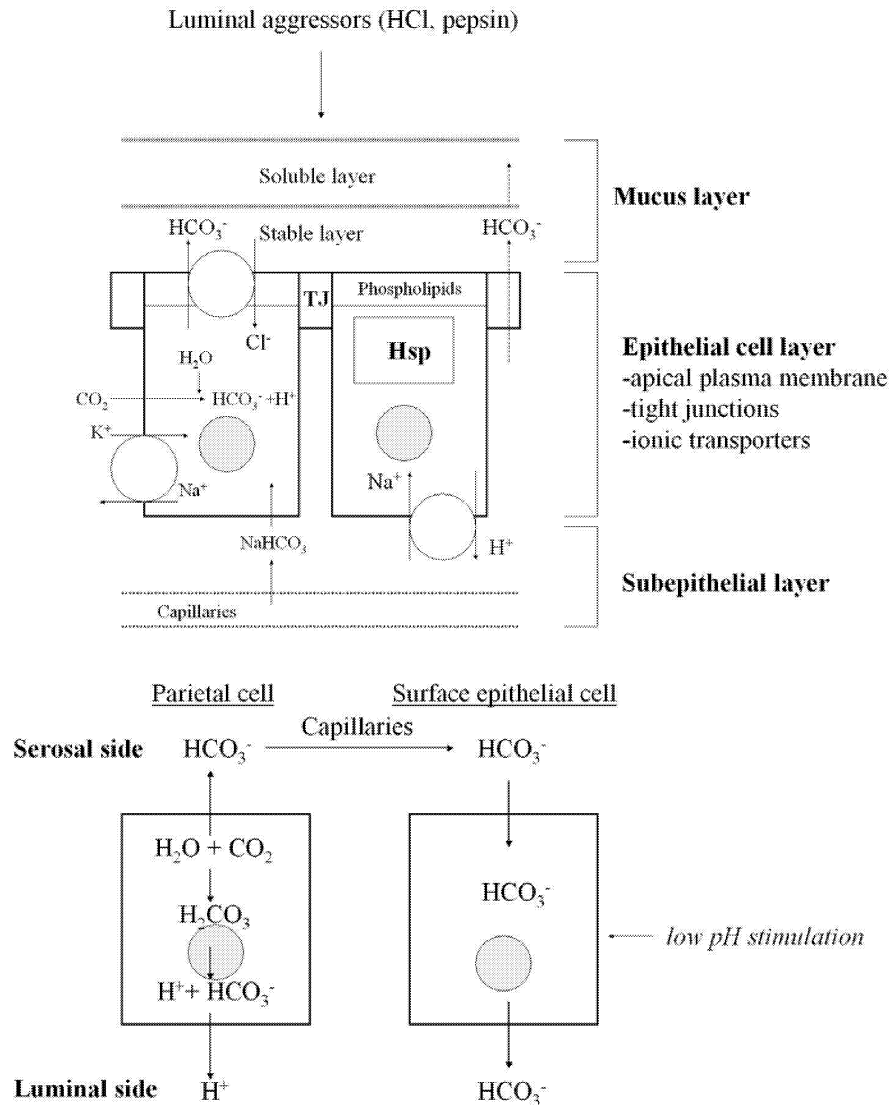


Figure 1. Schematic presentation of the gastric mucosal defense systems (mucosal barrier). Upper panel: ion transportes (open circles), passive diffusion via paracellular routes (interrupted arrows) and tight junctions (TJ). Lower panel: acid secretion in the parietal cells produces bicarbonate, which is transported via capillaries to the surface epithelial cells and eventually in to the lumen.

2.4 Heat shock response

Heat shock response denotes a characteristic and conserved response of a tissue to a stimulus, such as heat, by a complex cascade, the end-product of which is a family of evolutionarily conserved heat shock proteins (Morimoto, 1998, Morimoto and Santoro, 1998). In addition to its effect on protein synthesis, heat shock has been reported to induce activation of the arachidonic acid pathway, i.e. arachidonic acid release from the plasma membrane leading to increased prostaglandin and leukotriene synthesis (Calderwood et al., 1989). These eicosanoids, along with activation of the eicosanoid pathway, are critically involved in the regulation of synthesis of heat shock proteins (Jurivich et al., 1992, Jurivich et al., 1994, Lee et al., 1995, Jurivich et al., 1996).

Heat shock proteins (Hsps) function as molecular chaperones in normal and stressful conditions. Their expression has been reported in response to diverse chemical or physical stresses, including heat, ischemia, heavy metals, and toxins (Morimoto, 1998, Morimoto and Santoro, 1998). Hsps inhibit the aggregation of polypeptides and assist the folding and assembly of nascent polypeptides under normal conditions (McKay, 1993). Upon stressful conditions, Hsps improve cellular survival by repairing denatured proteins, especially in heat shocked cells, dissociating harmful protein aggregates, and regulating the correct folding and translocation of *de novo* synthesized proteins (Buchner, 1996, Morimoto and Santoro, 1998).

Hsps may be classified according to their molecular weight and function into distinct families (Table 1). Hsp90 family comprises two members, Hsp90 α and Hsp90 β (Hickey et al., 1989, Moore et al., 1989). Hsp90 forms an inactive complex with the steroid hormone receptor before steroid hormone binding and therefore participates in steroid hormone signaling (Richter and Buchner, 2001). The most important members of the Hsp70 family are Hsp70 and Hsc70. Hsp70, the major inducible heat shock protein, contains structures crucial for its chaperone activity: an ATPase domain, a substrate binding domain and a carboxy terminal motif that regulates the ATPase and substrate binding domains and co-chaperone interactions (McKay, 1993, Freeman et al., 1995, Fink, 1999). The subcellular localization is mainly cytosolic, but it may translocate to the nucleus also (McKay, 1993, Freeman et al., 1995, Fink, 1999). Hsp70 acts to minimize aggregation of newly synthesized proteins (Fink, 1999). Hsp70 is capable of inhibiting stress-induced apoptosis (Mosser et al., 2000). Heat shock cognate protein Hsc70 is constitutively expressed and acts as a chaperone in nonstressed cells (Benjamin and McMillan, 1998). It is involved in polymerization and disassembly of

clathrin coats in intracellular transport vesicles (Brodsky et al., 1991). The Hsp60 family contains only one member, Hsp60, which is stress-inducible and facilitates the protein folding process, unfolding and refolding of misfolded proteins (Fink, 1999). In contrary to Hsp70, Hsp60 is present in both mitochondria and cytosol. It also is important in the innate immune response (Vabulas et al., 2002). Hsp60 is also involved in mitochondrial protein biogenesis (Moseley, 2000, Voos and Rottgers, 2002). The family of small heat shock proteins includes Hsp27 and α B crystalline, which are capable of actin microfilament stabilization and inhibition of stress-induced apoptosis (Welsh and Gaestel, 1998). Characteristically, upon exposure to stress, small Hsps oligomerize to form large intracellular aggregates (Welsh and Gaestel, 1998).

Upon stressful stimuli, heat shock gene expression is regulated at both transcriptional and posttranscriptional levels. Transcriptional regulation is mediated by the stress-responsive heat shock transcription factor 1 (HSF1) (Wu, 1995). It is present in nonstressed cells in an inactive state (Wu, 1995), and becomes activated in response to stress, resulting in trimerization of HSF1 monomers, translocation of the trimers into the nucleus and binding to the promoter of heat shock genes (Baler et al., 1993, Sarge et al., 1993, Sarge, 1998). The intracellular pool of Hsp70 seems to exert negative feedback on HSF1 (Abravaya et al., 1992) (Figure 2). The threshold for HSF1 activation may be lowered and subsequent Hsp synthesis augmented by exposure to NSAIDs like indomethacin (Lee et al., 1995) or to arachidonic acid (Jurivich et al., 1994). However, these exposures differ, since arachidonic acid itself is capable of dose-dependently activating HSF1 leading to hsp synthesis. Treatment with indomethacin results in an incomplete heat shock response, but not Hsp synthesis (Lee et al., 1995). Heat shock releases arachidonic acid from the plasma membrane via activation of PLA₂ (Calderwood et al., 1989, Jurivich et al., 1996). An incomplete heat shock response characterized by activation of HSF1 without hsp synthesis may be induced by exogenous active PLA₂ (Jurivich et al., 1996) (Figure 2). Exogenous active PLA₂ also alters the temperature threshold for HSF1 activation (Jurivich et al., 1996) suggesting a key role in the activation of heat shock response by heat. In addition to the transcriptional regulation, a posttranscriptional mechanism mediated by hsp70 mRNA stabilization has been reported upon exposure to heat shock (Theodorakis and Morimoto, 1987) and hydrostatic pressure (Kaarniranta et al., 1998).

Table 1. Heat shock protein families of major importance

Protein family	Subcellular localization	Function
Hsp90 (Hsp90 α/β)	Cytoplasm/nucleus	Protein folding, transport, Steroid hormone signaling
Hsp70 (Hsp70/Hsc70)	Cytoplasm/nucleus	Protein folding, transport Inhibition of protein aggregation Inhibition of apoptosis Steroid hormone signaling Feedback regulation of Hsp70 Preconditioning-effect
Hsp60	Mitochondria	Protein folding
Small Hsps (Hsp27, α B crystallin)	Cytoplasm/nucleus	Actin filament stabilization Inhibition of apoptosis

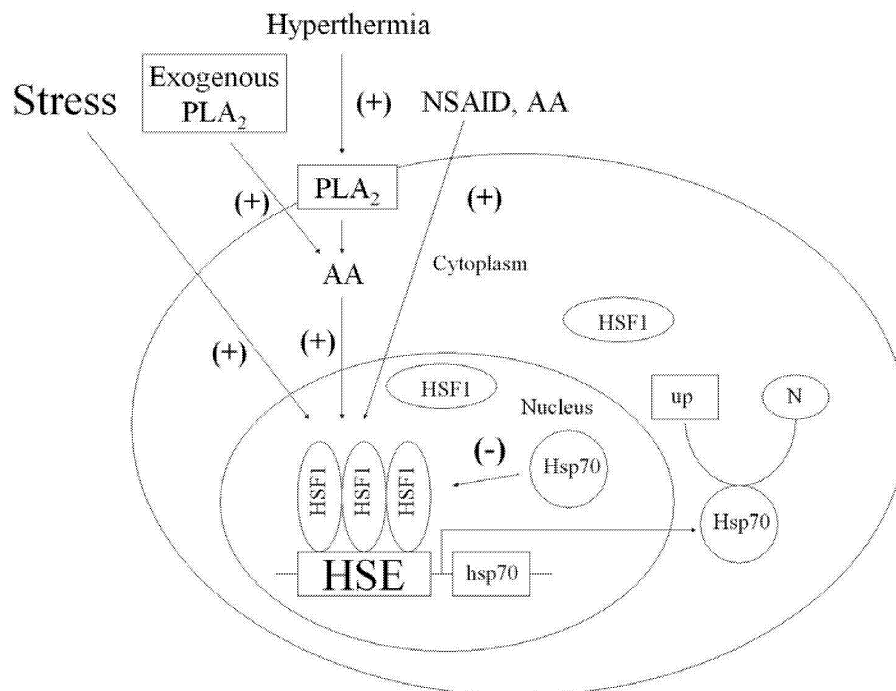


Figure 2. Illustration of HSF1 activation and subsequent hsp70 gene response. Upon stress, the HSF1 monomers trimerize and bind to the heat shock element (HSE) located in the hsp70 gene promoter area. In cytosol, Hsp70 protein interacts with unfolded

proteins (up), resulting in formation of functional proteins (N). Hyperthermia may activate HSF1 via activation of phospholipase A₂, resulting in release of arachidonic acid (AA) in the cytosol. Arachidonic acid activates HSF1. Exogenous AA and nonsteroidal anti-inflammatory drugs (NSAID) augment the activation of HSF1 by other stimulators.

2.5 Heat shock preconditioning and gastrointestinal mucosal injury

In heat shock preconditioning, heat shock protein production in the tissue is induced therapeutically to increase its tolerance to injury and to stimulate subsequent recovery (Benjamin and McMillan, 1998). Prolonged exposure to extreme stress may result in cell death, while transient stress results in the development of Hsp-mediated cytoprotection (Benjamin and McMillan, 1998). Heat shock preconditioning may be used to increase the threshold level needed to cause irreversible cellular injury. The preconditioning-effect requires elevation of Hsp expression in the absence of irreversible tissue injury. With respect to gastric pathophysiology, Hsp70 production has been demonstrated to increase the survival of cells exposed to various ulcerogenic factors, especially in models of deep gastric injury (Nakamura et al., 1991, Hirakawa et al., 1996, Itoh and Noguchi, 2000). Yet, there are few data about the role of heat shock response in gastric mucosal recovery after microscopic, i.e. superficial, injury.

Heat shock proteins are presumably responsible for increased cell survival under ischemia-reperfusion injury, especially in the myocardium (Benjamin and McMillan, 1998). Elevated expression of heat shock genes has been elucidated most extensively in the heart, liver, and kidney subjected to ischemia-reperfusion *in vivo* (Tacchini et al., 1993, Van Why et al., 1994, Nishizawa et al., 1996). In the small intestine, heat pretreatment, which induces expression of Hsp70, protects the small intestine against subsequent mucosal injury caused by ischemia-reperfusion in rats *in vivo* (Stojadinovic et al., 1995). In colonic epithelial cells *in vitro*, Hsp70 protects against oxidant induced injury of barrier function (Musch et al., 1999). Although there are data on the cytoprotective role of heat shock response in intestinal ischemia-reperfusion, the effects of ischemia and reperfusion *per se* on the small intestinal heat shock response have been poorly characterized.

2.6 Heat shock proteins and apoptotic cascades

Cellular damage may ultimately result in either necrotic or apoptotic cell death. The signaling mechanisms involved in apoptotic cell death are complicated and depend on the inducer, but most of them converge into the caspase pathway. Caspases are cysteine aspartic acid-specific proteases, which are ultimately responsible for the ultrastructural and biochemical apoptotic changes (Cohen, 1997, Thornberry and Lazebnik, 1998, Earnshaw et al., 1999, Nicholson, 1999, Hengartner, 2000). Caspase activation is characterized by a sequential cascade involving activation of upstream initiator caspases, which in turn activate downstream effector caspases. The signal for apoptosis may be transduced via 1) a death receptor-mediated pathway, in which tumor necrosis factor (TNF) ligand-receptor interaction leads ultimately to activation of initiator caspase-8 (Boldin et al., 1996, Muzio et al., 1996) or via 2) a mitochondrial pathway involving the release of cytochrome c (cyt c) to the cytosol and the formation of apoptotic protein complex leading to activation of initiator caspase-9 (Li et al., 1997, Zou et al., 1997). These pathways ultimately converge in the activation of effector caspase-3, resulting in morphological changes characteristic of apoptotic cells (Thornberry and Lazebnik, 1998) (Figure 3).

NSAIDs inhibit proliferation and induce apoptosis in gastric cancer cell lines (Zhu et al., 1999). Apoptosis in response to indomethacin in gastric cells is mediated through up-regulation of protein bax and activation of caspase-3 (Zhou et al., 2001). Bax is a proapoptotic protein belonging to the Bcl-2 protein family (Oltvai et al., 1993, Antonsson, 2001). It translocates from a cytoplasmic location to the mitochondria upon induction of apoptosis (Wolter et al., 1997), and induces release of cytochrome c into the cytosol (Rosse et al., 1998) (Figure 3). NSAIDs induce alterations in the eicosanoid pathways utilizing plasma membrane arachidonic acid as their substrate. Arachidonic acid pathways, on the other hand, are associated with regulation of tissue proliferation (Shimakura and Boland, 1992). A mild heat shock has also been found to induce G1 transition of cells, indicating that temperature may regulate tissue proliferation (Han et al., 2002).

Heat shock proteins have an intrinsic ability to confer protection against most apoptotic stimuli (Buzzard et al., 1998, Welsh and Gaestel, 1998, Jaattela, 1999, Mosser et al., 2000, Garrido et al., 2001). Both impaired or excessive Hsp70 can result in disturbances of growth control and ultimately cell death (Nollen and Morimoto, 2002). Both Hsp70 and Hsc70 are capable of inhibiting apoptosis by inhibiting release of

cytochrome c, processing of pro-caspase-9 and activation of initiator caspases (Mosser et al., 2000) (Figure 3). Heat stress results in inhibition of effector caspases upon subsequent heat shock (Mosser et al., 1997), and thereby confers protection against heat-induced apoptosis. Hsp70 is capable of rendering cells resistant to cell death induced by TNF (Jaattela et al., 1992) and caspase-3 overexpression (Jaattela et al., 1998). Hsp70 is capable of rescuing cells from apoptosis after the activation of effector caspases (Jaattela et al., 1998) (Figure 3). Hsp60 closely interacts with caspase-3 (Xanthoudakis et al., 1999, Samali et al., 1999), and a decrease in Hsp60 levels results in activation of caspase-3 and apoptosis (Kirchhoff et al., 2002) (Figure 3). Hsp60 also interacts upstream of caspase-3 activation, since it complexes with Bax (Kirchhoff et al., 2002).

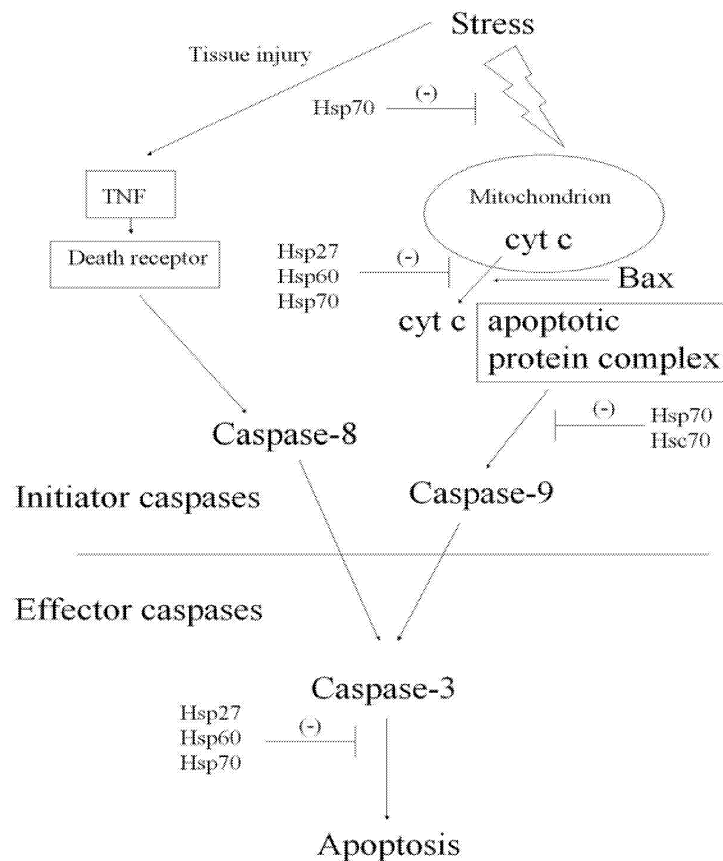


Figure 3. Cellular stress may result in mitochondrial injury characterized by leakage of cytochrome c (cyt c) into cytosol and formation of an apoptotic protein complex

resulting in the activation of caspase-9. On the other hand, tissue injury may result in release of tumor necrosis factor (TNF) which binds to the death receptor resulting in activation of caspase-8. The activation of initiator caspases results in activation of effector caspases such as caspase-3. Heat shock proteins may block the execution of apoptosis at several points (Hsps). On the other hand, Bax, a proapoptotic protein may promote apoptosis via stimulation of cytochrome c release from mitochondria (Modified from Jaattela, 1999).

3. AIMS OF THE PRESENT STUDY

The aims of the present study were to investigate:

1. The effect of heat shock preconditioning on gastric mucosa, its tolerance to and recovery from superficial injury in simulated conditions *in vitro*
2. The effect of heat shock preconditioning on tissue proliferative and apoptotic markers in simulated conditions *in vitro*
3. The mechanism of heat shock preconditioning-mediated modulation of apoptosis by analysis of caspase activity in simulated conditions *in vitro*
4. The effects of ischemia and reperfusion on intestinal heat shock response *in vivo*

4. MATERIALS AND METHODS

4.1 Experiments in guinea pig gastric mucosa *in vitro*

4.1.1 Ussing chamber perfusion (I-IV)

Guinea pig (I N=20, II N=60, III N=65, IV N=65) stomachs were removed and the fundic mucosa isolated and divided into two equal halves. The halves were randomized to experiment and control tissues, and mounted and perfused in neutral conditions in separate Ussing-chambers simultaneously as described in I-IV (Figure 4). The experiment tissues were exposed to heat shock preconditioning and normothermic recovery, while the control tissues were perfused under normothermic conditions. A superficial injury by hyperosmolar NaCl was induced and the tissues were allowed to recover (Figure 5) as described in I-IV. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Kuopio.

4.1.2 Modulation of Hsp synthesis and eicosanoid pathways (I-IV)

Depending on the experiment, the gastric mucosal explants were exposed to various pharmacological modulations (Figure 5). Exogenous arachidonic acid was added as described in I-IV. Arachidonic acid release and metabolism via lipoxygenases were inhibited with quercetin (Q) as described in I-IV. Arachidonic acid metabolism via cyclooxygenases was inhibited with indomethacin (IN) as described in I-IV. Total protein synthesis was inhibited with cycloheximide (CHX) as described in I-IV.

4.1.3 Electrophysiology (I, II)

During the experiment, the transmucosal electrophysiologic resistance (R) was determined as described in I-II by a current clamp method (Figure 6). The R was calculated according to Ohm's law. R after heat shock was defined as the ratio between R at 30 min after beginning of heat shock and baseline immediately before the heat shock. The recovery of R after superficial injury was defined as the ratio between R after 180 min restitution and the baseline value before superficial injury. The values are presented as percentage change relative to simultaneous normothermic controls.

4.1.4 Histology and morphometry (I, II)

After the experiment, the gastric mucosal explant tissue mounted in the Ussing chamber was fixed immediately *in situ* with formalin and embedded in paraffin. Tissue sections were cut and stained as described in I-II. The slides were analyzed to assess the progression of restitution as described in I-II.

4.1.5 Immunohistochemistry (III)

The gastric mucosal explant tissue was fixed with formalin and processed as described in the previous paragraph. Immunohistochemical analysis of Mib-1 and Bax expressions were performed as stated in III. The occurrence of positively stained cells in a gastric pit was recorded. The height of the mucosa in three randomly selected locations in a tissue section was measured. No difference between experiment and control tissues was found excluding the effect of heat shock on mucosal thickness.

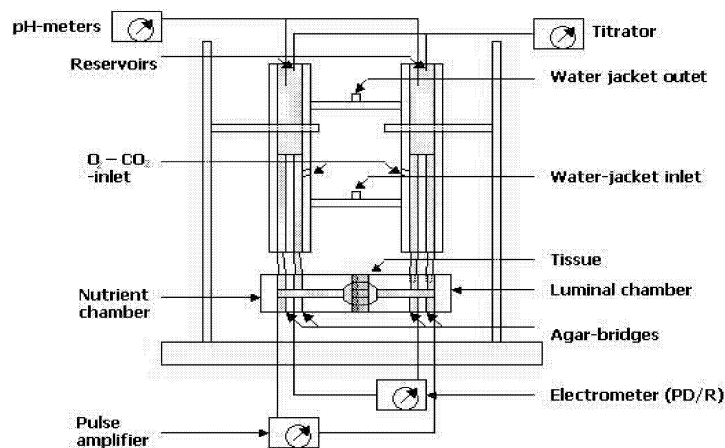


Figure 4. The Ussing chamber perfusion system.

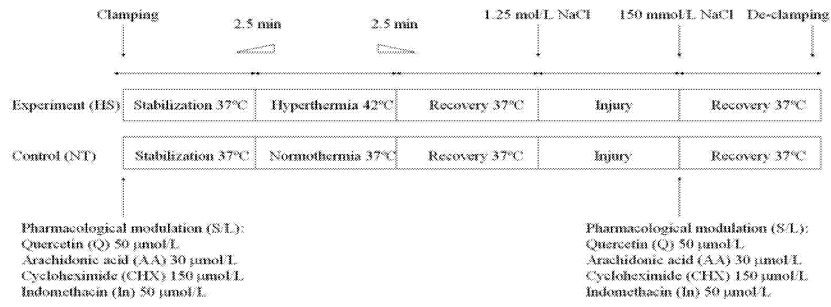


Figure 5. The experimental protocol

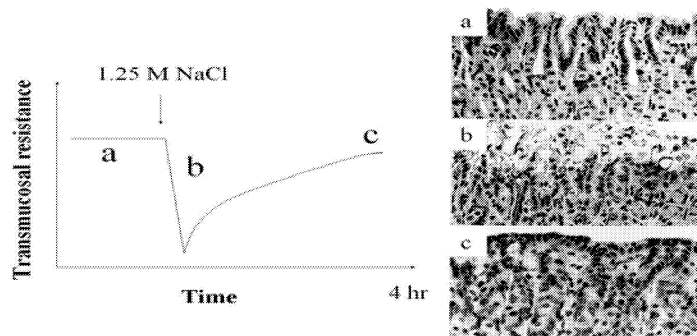


Figure 6. Monitoring of tissue recovery from superficial injury by measurements of transmucosal electrophysiologic resistance: a) before injury, b) immediately after superficial injury by 1.25 M NaCl and c) after 4 h restitution.

4.1.6 Western blot analysis (I, II, IV)

The gastric mucosal explant tissue was homogenized, and whole cell extracts were made described in I, II, IV and V. Protein extracts were electrophoresed on SDS/PAGE and transferred to a membrane as shown in I, II, IV and V. The membranes were probed with antibodies recognizing either heat shock protein 70 (Hsp70), heat shock protein 60 (Hsp60) or the heat shock cognate protein 70 (Hsc70). Thereafter, the films were exposed and developed and signals quantitated as described in I, II, IV and V.

4.1.7 Analysis of caspase-3 activity (IV)

Whole cell extracts from gastric mucosal explants were prepared as described in 4.6. Caspase-3 like activity was measured as shown in IV. The caspase-3 like activity was verified using a specific inhibitor of caspase-3, N-acetyl-Asp-Glu-Val-Asp-Aldehyde (Ac-DEVD-CHO).

4.1.8 Statistical analysis (I-IV)

Wilcoxon's signed ranks test, Mann-Whitney's U-test and Chi-square test were used. Spearman's rank correlation was used. ANOVA with Bonferroni's or Tamhane's post hoc test for multiple comparisons were used to analyze drug effects. Data are presented as means \pm SEM. A P value less than 0.05 was considered significant.

4.2 Experiments in pigs *in vivo*

4.2.1 Surgical procedures (V)

Female pigs (V N=4) were medicated and prepared as described in V. A tracheostomy was performed and the animals were mechanically ventilated. Arterial blood gas monitoring and the end tidal CO₂ fraction were monitored. An arterial catheter was inserted and a Swan-Ganz thermodilution catheter was installed. Systemic and pulmonary hemodynamics were recorded continuously. The core temperature was maintained \pm 0.5°C of baseline. A midline laparotomy was performed, the root of the superior mesenteric artery (SMA) was exposed and a precalibrated ultrasonic flow probe was placed around SMA for blood flow monitoring. A catheter was placed into the urinary bladder. Microdialysis capillaries with a polysulfone membrane were attached alongside a nasogastric tonometric catheter balloon and inserted into the lumen

of the jejunum through an enterotomy. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Kuopio.

4.2.2 Blood sampling (V)

Blood samples were aspirated from the femoral artery and a blood gas analyzer was used to determine arterial blood partial pressures of oxygen and carbon dioxide (apO_2 , $apCO_2$) and arterial blood oxygen saturations.

4.2.3 Microdialysis and tonometric measurements (V)

The presence of jejunal ischemia and reperfusion were monitored with intestinal luminal microdialysate lactate and a semi-automatic gas tonometer. The mucosal-arterial pCO_2 gradient ($dpCO_2$) of the small intestine was determined using the following equation: $dpCO_2 = mpCO_2 - apCO_2$, where $mpCO_2$ is the mucosal pCO_2 value.

4.2.4 Experimental design (V)

After a stabilization period an antimesenteric tissue biopsy was taken from the jejunal segment and, exposed to either a heat shock or normothermia and incubated *ex vivo* to provide either a heat-shocked positive control explant or negative control. The SMA was occluded and time series small intestinal samples were taken by open biopsy antimesenterically at 30 min intervals from the jejunal segment.

4.2.5 Histology (V)

Biopsy samples from the jejunum were processed as described previously

4.2.6 Western blotting (V)

The mucosal layer was separated from the seromuscular layer of the biopsy samples. Thereafter, the processing (homogenisation, centrifugation) was made as described earlier with the exception that only Hsp70 and Hsc70 were analyzed.

4.2.7 Northern blotting (V)

Biopsy samples from the jejunum were prepared as described previously. Total cellular RNA was isolated. Total RNA was separated on agarose/formaldehyde gel, transferred to a membrane and hybridized with isotope-labeled plasmids specific for

human hsp70 (Wu et al., 1985) and β -actin (Gunning et al., 1983). The signals were quantitated.

4.2.8 Gel mobility shift assay (V)

The protein extracts were prepared and mixed with isotope-labeled probes corresponding to the two overlapping heat shock elements (HSE) (Mosser et al., 1988). Protein-DNA complexes were resolved on a nondenaturing polyacrylamide gel. The specificity of the binding was ascertained by competition analysis with excess unlabeled probe. The presence of HSF1 was confirmed by supershift analysis.

4.2.9 Statistical analysis (V)

Friedman's nonparametric test for repeated measurements with Monte Carlo simulations was used. Data are presented as median and range (minimum-maximum), and $P < 0.05$ was considered significant.

5. RESULTS

5.1 The effect of heat shock preconditioning on gastric mucosa *in vitro* (I-IV)

Heat shock preconditioning of isolated quinea pig gastric mucosa induced a transient and significant increase in transmucosal resistance, increased significantly the expression of Hsp70 and Hsp60, and decreased significantly the Mib-1/Bax ratio. Caspase-3 activity was unaffected by heat shock (Table 2) and no morphological injury was observed (I, II). Based on these findings, this protocol of heat shock preconditioning was considered adequate, i.e. capable of inducing expression of Hsp70 but without unwanted side effects, i.e. disruption of the epithelial barrier either electrophysiologically or morphologically.

The change in transmucosal resistance by heat shock became negative rather than positive in all the experiments where the tissues were exposed to CHX alone or in combinations with other modulations (Table 2) (I, II). A similar effect was observed with Q, IN+Q, IN+AA, or IN+AA+Q (Table 2) (I, II). Exposure to AA, AA+Q or IN stabilized the transmucosal resistance during heat shock, upon which no significant changes were observed (Table 2) (I, II).

Exposure to heat shock preconditioning significantly decreased recovery of transmucosal resistance after superficial injury. This was sensitive to CHX in all the experiments (Table 2) (I, II). The heat shock induced inhibition of recovery of transmucosal resistance was also sensitive to Q, AA, AA+Q, IN, IN+AA or IN+AA+Q (Table 2) (I, II). Exposure to IN+Q was ineffective and seemed to augment the inhibition of recovery after superficial injury (Table 2) (II).

Heat shock preconditioning increased significantly Hsp70 expression in the gastric mucosa and this was sensitive to CHX in all the experiments (Table 2) (I, II). Superficial injury did not modulate the effect of heat shock. Exposure to IN+Q significantly augmented heat shock preconditioning induced expression of Hsp70 and significantly upregulated it also in the normothermic control exposed to IN+Q (Table 2) (I, II). Exposure to IN+AA or IN+AA+Q rendered expression of Hsp70 independent of heat shock since levels were elevated in both the heat shocked and normothermic tissues and significantly higher than those in native tissues (Table 2) (I, II).

The ratio of Mib-1/Bax was significantly decreased by heat shock preconditioning and blocked by CHX, AA, IN+Q, IN+AA, IN+AA+Q and IN+AA+CHX (Table 2) (III).

Exposure to AA+Q or IN reversed the effect of heat on Mib-1/Bax ratio positive (Table 2) (III). Superficial injury did not modulate these parameters.

Caspase-3 activity was not affected by heat shock preconditioning alone (Table 2) (IV). Superficial injury did not modulate caspase-3 activity either. Upon exposure to IN+Q, heat shock decreased significantly caspase-3 activity whereas upon exposure to IN+AA+CHX heat shock increased significantly caspase-3 activity (Table 2) (IV). With respect to drug effects, Q, CHX, IN+AA, IN+AA+Q significantly decreased caspase-3 activity in both the heat-shocked and normothermic tissues. Exposure to arachidonic acid together with quercetin increased significantly caspase-3 activity (IV).

Heat shock preconditioning significantly upregulated Hsp60 synthesis, and this was sensitive to CHX in all the experiments. Exposure to IN+AA or IN+AA+Q was capable of blocking Hsp60 expression.

Table 2. The effect of heat shock on various parameters

	Uninjured	N	Q	CHX	AA	AAQ	AACHX
R after HS	<u>16 %</u>	<u>16 %</u>	<u>-52 %</u>	<u>-40 %</u>	2 %	-4 %	<u>-15 %</u>
Recovery of R		<u>-8 %</u>	-2 %	3 %	-1 %	5 %	-4 %
Hsp70	<u>63 %</u>	<u>80 %</u>	<u>100 %</u>	14 %	<u>87 %</u>	<u>177 %</u>	-4 %
Mib-1	<u>-40 %</u>	<u>-33 %</u>	<u>-30 %</u>	-1 %	<u>-15 %</u>	<u>36 %</u>	-4 %
Bax	<u>-13 %</u>	4 %	7 %	<u>-15 %</u>	-16 %	<u>-36 %</u>	<u>38 %</u>
Mib-1/Bax	<u>-31 %</u>	<u>-35 %</u>	<u>-38 %</u>	16 %	4 %	<u>120 %</u>	<u>-30 %</u>
Caspase-3	1 %	-1 %	3 %	0 %	0 %	0 %	0 %
Hsp60	<u>69 %</u>	<u>77 %</u>	<u>104 %</u>	6 %	<u>85 %</u>	<u>192 %</u>	-39 %
	IN	INQ	INCHX	INAA	INAAQ	INAACHX	
R after HS	-8 %	<u>-73 %</u>	<u>-23 %</u>	<u>-54 %</u>	<u>-59 %</u>	<u>-23 %</u>	
Recovery of R	-5 %	<u>-20 %</u>	9 %	-4 %	6 %	-1 %	
Hsp70	<u>161 %</u>	<u>189 %</u>	8 %	4 %	-4 %	-3 %	
Mib-1	2 %	0 %	-10 %	1 %	-16 %	<u>12 %</u>	
Bax	<u>-36 %</u>	14 %	<u>33 %</u>	6 %	-5 %	<u>-17 %</u>	
Mib-1/Bax	<u>63 %</u>	-16 %	<u>-34 %</u>	-8 %	-13 %	32 %	
Caspase-3	1 %	<u>-54 %</u>	0 %	0 %	-1 %	<u>15 %</u>	
Hsp60	<u>161 %</u>	<u>91 %</u>	11 %	5 %	-6 %	-27 %	

The values are presented as percentage difference between heat shocked and normothermic tissues caused by exposure to heat shock and based on data in I-IV. Unmodulated tissue (N), exposure to quercetin (Q), cycloheximide (CHX), arachidonic acid (AA) or indomethacin (IN). Statistically significant changes are underlined and in bold.

5.2 The heat shock response in ischemia-reperfusion of small intestine *in vivo* (V)

Occlusion of SMA resulted in significant elevation of intestinal microdialysate lactate and the mucosal-arterial pCO₂ gradient, whereas reperfusion resulted in normalization of these indices (V, Fig 1). Reperfusion of pig small intestine resulted in a marked heat shock response, characterized by an induction of HSF1 DNA-binding activity and increased levels of hsp70 mRNA that corresponded to accumulation of Hsp70 protein both in the mucosa and muscularis (V, Fig 3, 4, 5). Ischemia induced a morphological mucosal injury (V, Fig 2) but had no effect on the jejunal heat shock response either in the mucosa or muscularis (V, Fig 3, 4, 5). Exposure of jejunal control explants *ex vivo* to 42°C heat shock resulted in a classical heat shock response characterized by acquisition of HSF1 DNA-binding activity, increased hsp70 mRNA levels and accumulation of Hsp70 both in the mucosa and muscularis (V, Fig 3, 4, 5).

6. DISCUSSION

6.1 The effect of heat shock on uninjured gastric mucosa (I-IV)

In uninjured mucosal explants, preconditioning was capable of transiently inducing a significant increase in transmucosal resistance and the expression of heat shock proteins Hsp70 and Hsp60 in the absence of a morphologic injury. However, this protocol resulted in suppression of cell proliferation as demonstrated by significantly decreased expression of Mib-1 and Mib-1/Bax ratio without alterations in caspase-3 activity. These findings indicate that the present protocol of heat shock preconditioning is adequate, i.e. causing significant upregulation of heat shock protein synthesis without evidence of unwanted side effects such as caspase-3 activation.

The transient increase in transmucosal resistance by heat shock, an index reflecting overall epithelial integrity (Svanes et al., 1982, Rutten and Ito, 1983, Critchlow et al., 1985), may indicate a decrease in epithelial permeability, possible due to the modulation of tight junction assembly (Moseley et al., 1994, Sussman and Renfro, 1997). Transmucosal resistance is dependent on resistance of the mucus and permeability of the epithelial layer, which is the sum of transcellular and paracellular components. Activation of acid secretion, altered composition of the bathing solution or altered permeability of the paracellular route may also result in changes in transmucosal resistance (Silen, 1988). Protein synthesis-dependent mechanisms presumably play a role in this phenomenon, since heat shock induced a significant transient decrease of transmucosal resistance when protein synthesis was inhibited paralleled with blockade of Hsp60 and Hsp70 synthesis and significantly decreased caspase-3 activity.

Exposure to arachidonic acid or to indomethacin stabilized transmucosal resistance during heat shock, suggesting that eicosanoid metabolism is also involved in this complex phenomenon. Indeed, heat shock releases arachidonic acid from plasma membrane, leading to activation of the arachidonic acid pathway (Calderwood et al., 1989, Jurivich et al., 1996). This effect, however, changed when the tissue specimens were exposed to either both of these agents simultaneously or to quercetin – the resistance transiently decreased during heat shock. Furthermore, upon simultaneous exposure to quercetin and arachidonic acid with or without indomethacin the transmucosal resistance remained stable. These results indicate that both indomethacin- and quercetin-sensitive eicosanoid pathways mediate the heat shock induced alterations of transmucosal resistance.

Heat shock significantly inhibited Mib-1 expression, and thus cell proliferation. This was not because of alterations in apoptotic activity, since Bax expression decreased significantly and caspase-3 activity was unaffected by heat shock. Heat shock has the capability to induce alterations in the proliferative balance, because it affects key enzymes, such as cyclin D1 critical for regulation of cell proliferation (Han et al., 2002). In addition, both impaired or excessive Hsp70 can result in disturbances of cell proliferation (Nollen and Morimoto, 2002). Whereas Mib-1 was localized in the proliferative neck region of gastric glands, as demonstrated earlier (Bhowmik et al., 1998), Bax was localized in the glandular base, as observed earlier in rat gastric mucosa (Neu et al., 2001). With respect to subcellular localization of the staining, Mib-1 staining was characteristically nuclear in accordance with a nuclear function (Scholzen and Gerdes, 2000). Bax staining was cytoplasmic indicating a cytoplasmic function (Wolter et al., 1997). To assess the effect of NaCl-injury, comparisons were performed between uninjured perfused tissues and superficially injured tissues. NaCl-injury itself did not affect either heat shock protein synthesis, Mib-1/Bax ratio or caspase-3 activity. The morphologic damage scores correlated significantly with the electrophysiologic recovery, indicating that transmucosal resistance can be used to characterize the progression of restitution.

In vitro experiments have been criticized because of isolation from the circulation and from nervous connections. *In vivo*, the effects of circulatory factors are mediated to the mucosa through capillaries. In the Ussing chamber model, physiological conditions are created and circulatory factors are simulated. The Ussing chamber model enables better control of variables than in more complex *in vivo* models and continuous monitoring of the electrophysiology, which is a pre-requisite for this type of research, where epithelial morphology is followed dynamically electrophysiologically instead from a static morphologic specimen.

6.2 The effect of heat shock preconditioning on mucosal recovery after superficial injury (I-IV)

The upregulation of Hsp70 and suppression of Mib-1/Bax ratio in the tissue by heat shock preconditioning was associated with significant impairment of both electrophysiological and morphological restitution after superficial injury by hyperosmolar sodium chloride. Suppression of cell proliferation may reflect an acute response to injury. Due to the limited time-scale, no further conclusions can be drawn.

Similarly, in gastric mucosal injury induced by water immersion and restraint stress in rats, a decreased rate of cell proliferation and an increased expression of Bax within 4 hours from the injury has been found (Konturek et al., 1999). Cell proliferation returned to normal values and expression of Bax declined within 24 h from the injury (Konturek et al., 1999). An increased intracellular pool of aggregating denatured proteins is the major inducer of Hsp70 accumulation (Benjamin and McMillan, 1998). The denaturation of structural proteins, which are needed for cellular adhesion, shape change and migration, is among the possible heat-induced alterations in a cell. These events are controlled by growth factors, which also control cellular proliferation. Heat shock may inactivate growth factors like FGF, EGF and TGF- β , which are critically involved in the regulation of restitution of both gastric and intestinal cells (Dignass and Podolsky, 1993, Paimela et al., 1993, Dignass et al., 1994, Riegler et al., 1996, Yanaka et al., 1996). Heat shock response and synthesis of growth factors are interrelated, since TGF- β regulates synthesis of Hsp70 and Hsp90 in cultured chicken embryo cells, whereas FGF and EGF are ineffective (Takenaka and Hightower, 1992). In cultured fibroblasts exposed to ultraviolet B irradiation, TGF- β mediates the upregulation of Hsp70 synthesis (Cao et al., 1999). These potential mechanisms may account for impaired electrophysiological and histological restitution in heat shock-preconditioned tissues. Another possible mechanism is through disturbance of the function of the mucoid cap. Heat may either activate acid secretion or interfere with mucus production or bicarbonate secretion and create an acidic micromilieu, therefore not allowing restitution to occur. A potential mechanism by which heat shock could exert these effects is via modulation of prostaglandin synthesis (Calderwood et al., 1989), which is a critical regulator of both mucus and bicarbonate secretion (Takeuchi et al., 1999, Tani et al., 2002).

Quercetin abolished the heat shock-induced inhibition of restitution and caspase-3 activity. It did not have any effect on either heat shock-induced suppression of Mib-1/Bax ratio or Hsp60 or Hsp70 synthesis. The net effect of quercetin was therefore to suppress apoptosis, at least partially explaining the beneficial effect on restitution. Quercetin is capable of inhibiting both release of arachidonic acid from plasma membrane (Fawzy et al., 1988, Lindahl and Tagesson, 1993, Hirasawa et al., 1995) and inhibiting leukotriene synthesis from arachidonic acid (Laughton et al., 1991, Kim et al., 1998, Bossu et al., 1999). Therefore, quercetin could affect the intracellular arachidonic

acid pool. Previously arachidonic acid has been shown to directly activate caspase-3 (Cao et al., 2000). Free radicals are critical inducers of apoptosis in cultured gastric epithelial cells (Kusuhara et al., 1999). Quercetin is also a potent flavonoid antioxidant capable of inhibiting reactive oxygen radical formation (Feng et al., 2001), which could in part explain the antiapoptotic potential of quercetin.

Arachidonic acid is critically involved in the protection of the gastric mucosa, especially in deep models of injury and during normothermic conditions (Hollander et al., 1982, Hollander and Tarnawski, 1986, Hollander and Tarnawski, 1991). Cyclooxygenases use intracellular arachidonic acid as a substrate to generate prostaglandins that are essential for gastric mucosal defense (Takeuchi et al., 1999, Takahashi et al., 2002). Restitution of either rat or guinea pig gastric mucosa is not dependent on the arachidonic acid pathway products, at least under normothermic conditions (Ito et al., 1984, Silen and Ito, 1985). In the present study, arachidonic acid abolished the heat shock-induced inhibition of restitution and suppression of cell proliferation. It did not, however, modulate the effect of heat shock on synthesis of Hsp60 and Hsp70 or caspase-3 activity. Previously, 30 $\mu\text{mol/L}$ arachidonic acid has been shown to augment the heat shock-induced expression of Hsp70 in cell culture models (Jurivich et al., 1994). Therefore, the effects of arachidonic acid on restitution or cell proliferation are not likely to be mediated via Hsp synthesis. When arachidonic acid release and leukotriene synthesis were modulated with quercetin upon exposure to arachidonic acid, heat shock significantly increased cell proliferation, paralleled by a slightly, but significantly increased caspase-3 activity. These findings were strikingly different from the experiments with quercetin alone. Simultaneous exposure to quercetin and arachidonic acid did not alter restitution. Addition of quercetin may result in shunting towards prostaglandin synthesis or lead to an increased intracellular pool of arachidonic acid or perturbation of membrane lipid turnover, which could eventually result in increased caspase-3 activity (Surette et al., 1996, Cao et al., 2000). With respect to the proliferative rate, a possible explanation is heat shock-induced synthesis of pro-proliferative prostaglandins. Previously, prostaglandins, especially E2 analogues, have been shown to regulate gastric epithelial cell proliferation, and eventually result in gastric mucosal hyperplasia (Uribe et al., 1988, Goodlad et al., 1990). In gastric epithelial cells, prostaglandin E2 activates the EGF receptor, which mediates the mitogenic signaling in gastric epithelial cells (Pai et al., 2002). The existence of pro-proliferative prostaglandins has been demonstrated earlier (Michiels et al., 1994). It

appears, that exogenously administered arachidonic acid may modulate the synthesis of prostaglandins induced by heat shock.

Indomethacin abolished the heat shock-induced inhibition of restitution. It reversed the heat shock-induced suppression of cell proliferation, increasing proliferation without any effect on caspase-3 activity. This indicates that heat shock might inhibit restitution and cell proliferation via indomethacin-sensitive pathways. Concentrations of indomethacin above 10 μM in human gastric cells *in vitro*, are capable of up to 99% inhibition of prostaglandin synthesis (Kokoska et al., 1998). Exposure to indomethacin may also lead to shunting towards leukotriene synthesis by lipoxygenases. High doses of indomethacin have been shown to increase the rate of cell proliferation in the rat oxyntic mucosa (Uribe et al., 1995). Depending on the dose, indomethacin has been shown to exert its effects on apoptosis via activation of caspase-3 and bax *in vitro* within 12 h from exposure to 400 μM indomethacin (Zhou et al., 2001) and *in vivo* within 2 hours from exposure to 60 mg/kg indomethacin (Slomiany et al., 1999). Analogous to pro-proliferative prostaglandins, leukotrienes have been found to induce proliferation in rat fibroblasts (Woo et al., 2002). Indomethacin seemed to augment the expression of Hsp70, which is in line with cell-culture studies, possibly due to increased intracellular arachidonic acid (Jurivich et al., 1992, Lee et al., 1995). In contrast, the levels of Hsp60 were not affected.

Upon exposure to indomethacin and quercetin, presumably leading to blockade of eicosanoid metabolism via cyclo-oxygenases and lipoxygenases, the heat shock-induced inhibition of electrophysiological recovery remained, whereas the suppression of cell proliferation was abolished. Morphological restitution was comparable to that of normothermic tissues. These findings suggest that there is interference with other mechanisms determining the transmucosal resistance and the gastric mucosal barrier, presumably mechanisms responsible for ion conductance and production of mucus, but not strictly epithelial migration. Interestingly, under these conditions heat shock significantly decreased caspase-3 activity. These observations differ strikingly from experiments with quercetin alone, indicating that an indomethacin-sensitive mechanism might be operative in caspase-3 regulation. These observations are in line with previous studies, in which preconditioning heat shock has been found to protect against effector caspase activation (Mosser et al., 1997). These pharmacological interventions may lead to an increased intracellular pool of arachidonic acid due to substrate accumulation – the effect of heat shock on Hsp70 expression was significantly augmented, while no effect

on synthesis of Hsp60 was found. This modulation resulted also in significant augmentation of Hsp70 expression in the normothermic control tissue. This suggests that these two separate chaperones are differentially modulated by eicosanoid-mediated mechanisms and may ultimately be induced without hyperthermia. Most importantly, Hsp70 has been previously considered to be the critical mediator of effector caspase inhibition (Mosser et al., 1997, Mosser et al., 2000). These results indicate that a certain threshold level for Hsp70 may exist for its antiapoptotic activity.

Combined exposure to indomethacin and arachidonic acid blocked the heat shock-induced inhibition of restitution. Compared to indomethacin alone, addition of arachidonic acid blocked the heat shock-induced increase of cell proliferation and induction of Hsp60. Further support for differential regulation of Hsp60 and Hsp70 by eicosanoid pathways is provided by the finding that levels of Hsp70 were equally elevated in both the heat-shocked and normothermic tissues, whereas levels of Hsp60 remained at baseline. The functions of Hsp70 and Hsp60 exhibit a remarkable difference. Hsp70 is the major inducible Hsp and is found mainly in the nucleus and cytosol, functioning to minimize aggregation of newly synthesized proteins (Fink, 1999). On the other hand, Hsp60 is localized both in mitochondria and the cytosol and functions to facilitate the protein folding process (Fink, 1999). Both of these proteins are capable of modulating caspase-3 activity – Hsp70 rescues cells from apoptosis after activation of the effector caspases (Jaattela et al., 1998), while Hsp60 has been shown to either inhibit caspase-3 (Gupta and Knowlton, 2002) or facilitate the maturation of pro-caspase-3 to its active form (Xanthoudakis et al., 1999). In contrast to earlier observations, simultaneous indomethacin and arachidonic acid treatment abolished caspase-3 activity, whereas neither indomethacin nor arachidonic acid alone was capable of that. This provides further support for the role of eicosanoids in the regulation of caspase-3 activity in the gastric mucosa. According to previous studies, caspase-3 activity reflects apoptotic activity, which on the other hand, has been shown to parallel the degree of gastric mucosal injury in rats *in vivo* (Piotrowski et al., 1997, Slomiany et al., 1997, Slomiany et al., 1999). Based on these findings, it is probable that treatments lowering caspase-3 activity can be considered as cytoprotective strategies against superficial injury. Indomethacin abolished the cell proliferation induced by heat shock in the simultaneous presence of arachidonic acid and quercetin. This finding provides strong support for the hypothesis that exposure to arachidonic acid and quercetin upon heat shock might result in synthesis of pro-proliferative prostaglandins

or some other effectors sensitive to indomethacin. It must be emphasized that both prostaglandins and leukotrienes are capable of increasing or decreasing the rate of cell proliferation (Shimakura and Boland, 1992). In addition to its effects on eicosanoid pathways, indomethacin can exert its actions by mechanisms independent of prostaglandins. For example, indomethacin modulates intracellular calcium homeostasis (Kokoska et al., 1998), which regulates arachidonic acid release from the plasma membrane (Preclik et al., 1992, Chulada et al., 1996). The importance of this mechanism is that both intracellular calcium (Tombal et al., 2002) and intracellular arachidonic acid (Cao et al., 2000) are key stimuli for apoptosis.

Protein synthesis-dependent mechanisms are critical in the heat shock-induced effects on gastric mucosa - upon exposure to cycloheximide, the heat shock-induced suppression of cell proliferation and inhibition of both morphological and electrophysiological restitution were abolished. Cycloheximide also blocked caspase-3 activity, which is in line with previous findings in guinea pig gastric mucous cells *in vitro* (Johal and Hanson, 2000). This indicates that *de novo* protein synthesis is required for these events. Cessation of protein synthesis was verified by blockade of Hsp60 and Hsp70 synthesis. These results are in parallel with the early observation in Ussing chambered frog gastric mucosa that protein synthesis is not needed for restitution to occur (Critchlow et al., 1985). The mechanism by which cycloheximide could act as a cytoprotective agent is based on the fact that via translational inhibition, there is also a decrease in potentially thermolabile nascent chains and polypeptides that could otherwise aggregate and exert cytotoxic effects (Michels et al., 2000). However, when the tissues were exposed to both arachidonic acid and cycloheximide, the heat shock-induced suppression of cell proliferation persisted. This might indicate that arachidonic acid is capable of regulating cell proliferation also through mechanisms independent of protein synthesis. An absolute contrast with experiments performed with indomethacin only was seen when the tissues were exposed to simultaneous indomethacin and cycloheximide - inhibition of protein synthesis reversed the promotion of cell proliferation to significantly suppressed proliferation. This suggests that the proliferative effect of indomethacin seems to be dependent on protein synthesis-dependent mechanisms. Simultaneous blockade of prostaglandin and *de novo* protein synthesis led to an increased rate of cell proliferation.

According to the present data, the role of heat shock proteins in the protection against gastric mucosal injury is different in superficial and deep models of injury. Hsp70 has

been shown to protect against deep mucosal injury (Hirakawa et al., 1996, Itoh and Noguchi, 2000). However, in the present study, Hsp70 did not seem to either accelerate repair of superficial mucosal injury or prevent the injury. This may be due to several factors that differ between superficial and deep models of mucosal injury. In deep mucosal injury induced by necrotizing agents, the repair process is characterized by necrosis, inflammation and scar formation. The repair process is critically dependent on cell proliferation under a period of multiple days. On the other hand, superficial injury is characterized by detachment of nonviable surface cells and followed by rapid epithelial restitution operating on a time scale of hours and independent on cell proliferation (Rutten and Ito, 1983). Deep necrotizing injury, such as the one induced by ethanol is itself an inducer of Hsp70 in gastric mucosal erosions *in vivo* in rats (Saika et al., 2000). However in the present study, no effect of hyperosmolar sodium chloride on hsp synthesis was found. These differences may explain why induction of Hsp70 itself was not associated with protection against superficial injury.

6.3 The effect of ischemia-reperfusion on intestinal heat shock response (V)

Based on control biopsies taken after stabilization, the surgical procedures did not influence the heat shock response. Occlusion of superior mesenteric artery and resulting ischemia in the small intestine was verified by measurements of intestinal luminal microdialysate lactate (Tenhunen et al., 1999) and the mucosal-arterial carbon dioxide gradient by tonometry (Landow and Andersen, 1994). Interestingly, upon restoration of blood supply, the lactate concentration significantly increased, which might indicate still continuing mucosal dysfunction. Tonometric measurements clearly verified restoration of blood supply, as shown in previous studies (Tenhunen et al., 1996, Heino et al., 1997, Tenhunen et al., 1999).

Ischemia did not result in activation of the heat shock response. Activation of the heat shock response occurred in the beginning of reperfusion. According to previous studies, pre-induction of Hsp70 protects against small intestinal ischemia-reperfusion injury in rats *in vivo*, while neither ischemia nor reperfusion itself seemed to activate the response (Stojadinovic et al., 1995). In rat heart *ex vivo*, reperfusion is the major inducer of heat shock response, as reflected by rapid activation of HSF1 and accumulation of hsp70 mRNA at the beginning of reperfusion (Nishizawa et al., 1996). Among the possible activators during reperfusion are oxygen free radicals, as shown in rat heart *ex vivo* (Nishizawa et al., 1999) and in intestinal epithelial cells *in vitro* (Gebhardt et al., 1999).

Rapid activation of HSF1 by reperfusion indicates that other activators may also be involved. Previously, HSF1 has been shown to be rapidly activated by arachidonic acid (Jurivich et al., 1994), synthesis of HSF1-activating prostaglandins (Amici et al., 1992), cytokines (Haglund, 1993, Tamion et al., 1997, Cuzzocrea et al., 1999) or changes in interstitial fluid osmolarity (Sheikh-Hamad et al., 1998), pH and cellular ATP content (Van Why et al., 1994). The interstitial osmolarity is likely to change rapidly, since in the present study, increased lactate concentration at the beginning of reperfusion coincided with activation of HSF1 followed by a rapid decline in lactate concentration. These changes may induce rapid fluxes in interstitial fluid pH and osmolarity.

Reperfusion did not cause apparent histological injury, which is in line with previous observations (Blikslager et al., 1997a). On the other hand, ischemia resulted in a characteristic mucosal injury. Reperfusion induced the heat shock response in both the mucosa and muscularis, although no evidence of histological injury was seen in the muscularis layer.

7. CONCLUSIONS AND SUMMARY

This study offers novel information about the gastric mucosal response to heat shock preconditioning and the effect of ischemia and reperfusion on the heat shock response in the small intestine.

1. Heat shock preconditioning of isolated guinea pig gastric mucosa in neutral conditions *in vitro* induced transient alterations in transmucosal resistance, synthesis of heat shock proteins without morphological injury and subsequent inhibition of restitution via mechanisms dependent on both protein synthesis and eicosanoid pathways.
2. Heat shock preconditioning suppressed proliferation via mechanisms dependent on protein synthesis and eicosanoid pathways.
3. Heat shock preconditioning modulated caspase-3 activity by a mechanism dependent on eicosanoid pathways. Independent of heat shock, caspase-3 activity could be modulated via mechanisms mediated through eicosanoid pathways and protein synthesis.
4. Reperfusion but not acute ischemia induced a transcriptionally mediated heat shock response in the small intestine *in vivo*.

Based on this thesis, hyperthermia may be utilized to produce alterations in the epithelial integrity, expression of heat shock proteins, apoptotic activity, cell proliferation and capability to reconstitute in isolated guinea pig gastric mucosa *in vitro*. These alterations are sensitive to modulation of protein synthesis and eicosanoid pathways. Reperfusion is a critical inducer of heat shock response in the small intestine *in vivo*.

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