THE ROLE OF THE HEAT SHOCK RESPONSE IN THE CYTOPROTECTION OF THE INTESTINAL EPITHELIUM

DE ROL VAN DE HEAT SHOCK RESPONS BIJ DE BESCHERMING VAN HET DARMEPITHEEL

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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Oh, the depth of the riches of the wisdom and knowledge of God!
How unsearchable his judgments,
and his paths beyond tracing out!
"Who has known the mind of the Lord?
Or who has been his counselor?"
"Who has ever given to God,
that God should repay him?"
For from him and through him and to him are all things.
To him be the glory forever! Amen.

Romans 11:33-36

to the always stressed primal patient,
the cell

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GENERAL INTRODUCTION

BENEFICIAL AND PATHOGENIC BACTERIA AS WELL AS FACTORS OF BACTERIAL ORIGIN INTERFERING WITH THE INTESTINAL EPITHELIUM

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The intestinal environment accommodates a wide range of contents ranging from harmless beneficial dietary and microbial flora to harmful pathogenic bacteria. This has resulted in the development of highly adapted epithelial cells lining the intestine. This adaptation involves the potential of crypt cells to proliferate and to constantly replace villous cells that are lost due to age, apoptosis or death. As a result, the normal intestinal epithelial integrity and functions are maintained. This phenomenon is eminent in the intestinal defence whereby the intestinal epithelial cells (IECs) serve a physical barrier function against luminal agents. The protection against agents in the gut lumen can only be effective if the epithelium is intact. Restitution of the damaged epithelium is therefore crucial in this type of defence.

Apart from being a physical barrier, IECs produce immune mediators such as inflammatory cytokines and heat shock proteins (Hsps) in response to pathogens, adverse conditions, and various forms of stress[1-4]. These mediators are aimed at eliminating the pathogens and protecting the cells by rescuing the already synthesized proteins, respectively. However, persistent production of proinflammatory cytokines often causes chronic inflammation followed by damage to the epithelium. In most cases therefore, their production is down regulated after resolving the inflammation. This down-regulation is mediated, at least in part, by way of Hsp production[5-7]. The expression of Hsps by IECs could henceforth, be part of a protective mechanism against stress, infection or inflammation of the intestinal epithelium. Though their actual mechanisms are not fully known, Hsps interfere with the activities of activators of several genes. These activators include the nuclear factor kappa B (NF-κB) and mitogen activated protein kinases (MAPK) that are involved in the production of inflammatory, growth and stress mediators[1,7].

The common intestinal luminal contents like dietary products and intestinal microflora substantially influence the cytoprotection of the IECs. For instance, the bacterial microflora ferment dietary components to yield biologically active products. In particular, short chain fatty acids (SCFAs), that are the fermentation products of carbohydrates[8], are constantly found in the colon of animals at various concentrations[9,10]. Their presence in the human colon affects important biological processes such as growth, metabolism and differentiation of the IECs[11,12]. These processes are vital in maintaining the intestinal barrier integrity that separates and thus protects the sterile host milieu from the luminal environment. For example, induction of differentiation is associated with an increased transepithelial resistance. Such resistance may account for the inhibition of the pathogenic invasion of differentiated as opposed to undifferentiated IECs[13].

While ample evidence is available to asseverate the essential role of SCFAs to the intestinal immune system[14-20], it is unknown whether this effect involves Hsps. Several studies have linked SCFAs with the alleviation of

inflammatory bowel diseases such as ulcerative colitis and Crohn's disease in humans[19,20]. Their deficiency exacerbates the development of these diseases whilst the restoration of the normal endogenous concentrations by intracolonic infusion can improve the conditions. Though the exact mechanism is enigmatic, SCFAs, in particular butyrate, affect the IEC production of inflammatory cytokines that are pivotal to inflammation[14,15,17]. Butyrate also has the potential to induce the expression of Hsps in various mammalian cells including the IECs[16]. Bearing in mind the anti-inflammatory role of Hsps, it is tempting to suggest that part of the anti-inflammatory role of butyrate is via production of Hsps.

At times of infection, Hsps may play a role in cellular inhibition of pathogenic invasion. This can be possible through stabilization of the cell cytoskeleton. In human IECs, Hsps have been observed to protect the integrity of the actin cytoskeleton against oxidant-induced injury[21]. In other systems Hsps play a role in the formation and function of the eukaryotic cell cytoskeleton[22]. Their mechanism involves stabilization of the actin filaments by cross-linking[23]. In doing so, Hsps could prevent invasion of pathogens. Intestinal pathogens that distort the cell membranes by causing ruffles during invasion (Fig 1.1) may induce the production of Hsps as a response to stabilize the actin filaments and subsequently hamper further invasion.

It is well apprehended that the colonization of the intestinal milieu by beneficial bacteria (among others *Lactobacillus* spp) is stable over time, implying that they multiply in a particular niche at a rate that equals or exceeds their rate of washout or elimination at the site. This microflora may become firmly associated to the intestinal wall[24] and thus may affect cellular responses to various stimuli. Part of their beneficial effect is that of reducing establishment of pathogenic bacteria such as *Salmonella*[25], a phenomenon called competitive exclusion[26]. As to whether the microflora has additional beneficial effects with regard to production of Hsps and modulation of inflammatory cytokine production remains cryptic.

It is interesting to explore the various ways through which Hsps protect the intestinal epithelium. The work explained in this thesis was aimed at establishing the basis of that protection. Its scope is limited to the effects of constantly beneficial and transiently pathogenic intestinal components on the induction of Hsps. This protection can range from inhibition of invasion by pathogens through repair of the cytoskeleton, maintenance of the barrier integrity through restitution of the damaged epithelium, or suppression of proinflammatory cytokines, especially the chemokines that are pivotal to the development of inflammation.

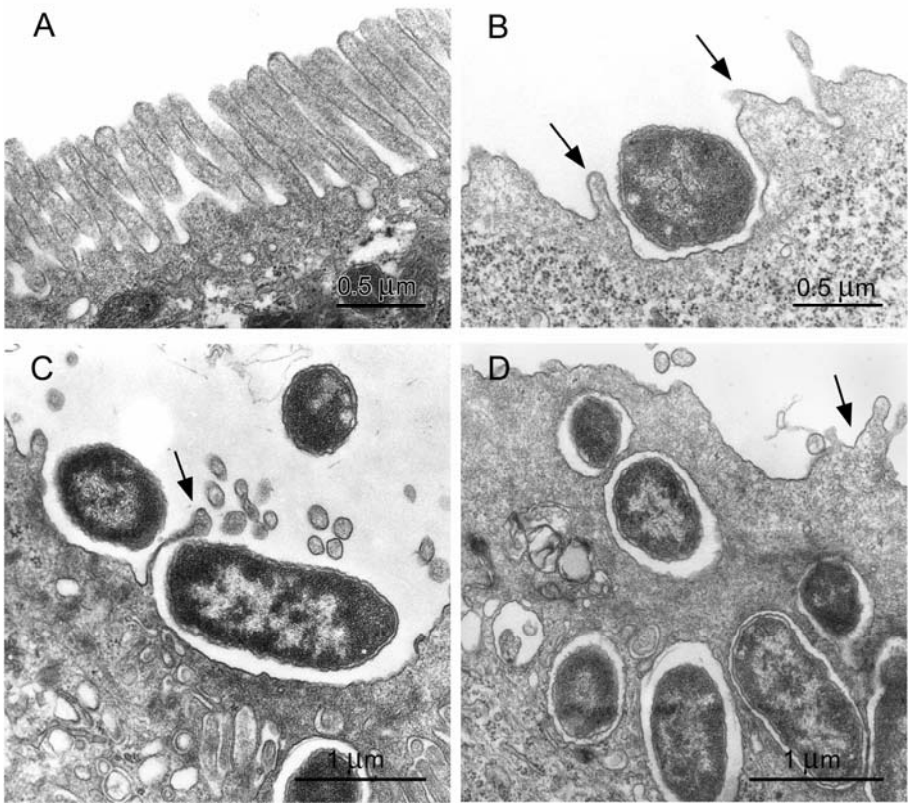

Fig 1.1. Adherence to and invasion of differentiated Caco-2 cells by Salmonella enteritidis 857. *Differentiated cells grown on tissue culture inserts (pore size 0.4 µm; growth area 1 cm^2) were incubated for 0 (A) (control), 10 (B) or 60 (C and D) min with 1 ml plain serum- and gentamicin-free cell culture medium containing 10^8 bacteria (200 bacteria/Caco-2 cell). Arrows indicate the presence of membrane ruffles.*

Specifically, it explored the interference of Hsps with NF-κB and MAPK pathways in down regulating production of proinflammatory cytokines (Chapter 2), the benefits of SCFA-induced modulation of biological processes, especially the intestinal epithelial growth and barrier integrity, against *Salmonella enteritidis* invasion (Chapter 3), whether the anti-inflammatory role of SCFAs in intestinal inflammatory diseases can be mediated through production of Hsps (Chapter 4),

the potency of *S. enteritidis* to induce Hsp expression by IECs (Chapter 5), and as to whether the protective role of *Lactobacillus* spp to the intestinal cells involves the synthesis of Hsps (Chapter 6).

References

1. Hobbie S, Chen LM, Davis RJ, Galan JE. 1997. Involvement of mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by *Salmonella typhimurium* in cultured intestinal epithelial cells. *J Immunol* 159: 5550-5559.
2. Awane M, Andres PG, Li DJ, Reinecker HC. 1999. NF-κB-inducing kinase is a common mediator of IL-17, TNF-α-, and IL-1β-induced chemokine promoter activation in intestinal epithelial cells. *J Immunol* 162: 5337-5344.
3. Elewaut D, DiDonato JA, Kim JM, Truong F, Eckmann L, Kagnoff MF. 1999. NF-κB is a central regulator of the intestinal epithelial cell innate immune response induced by infection with enteroinvasive bacteria. *J Immunol* 163: 1457-1466.
4. Ovelgönne JH, Koninkx JFJG, Pusztai A, Bardocz S, Kok W, Ewen SWB, Hendriks HGCJM, van Dijk JE. 2000. Decreased level of heat shock proteins in gut epithelial cells after exposure to plant lectins. *Gut* 46: 679-687.
5. Cahill CM, Waterman WR, Xie Y, Auron PE, Calderwood SK. 1996. Transcriptional repression of the proinflammatory 1β gene by heat shock factor 1. *J Biol Chem* 271: 24874-24879.
6. Chu EK, Ribeiro SP, Slutsky AS. 1997. Heat stress increases survival rates in lipopolysaccharide-stimulated rats. *Crit Care Med* 25: 1727-1732.
7. Yoo C, Lee S, Lee C, Kim YW, Han SK, Shim Y. 2000. Anti-inflammatory effect of heat shock protein induction is related to stabilization of IκBα through preventing IκB kinase activation in respiratory epithelial cells. *J Immunol* 164: 5416-5423.
8. Cummings JH, Branch WJ. 1990. Fermentation and production of short chain fatty acids in human large intestine. In: *Dietary Fiber: Basic and Clinical Aspects*, ed Vahouny GB, Kritchevesky D. Plenum, NY, 131-152.
9. Cummings JH, Pomare EW, Branch WJ, Naylor CPE, MacFarlane GT. 1987. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* 28: 1221-1227.
10. Treem WR, Ahsan N, Shoup M, Hyams J. 1994. Fecal short-chain fatty acids in children with inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 18: 159-164.
11. Bernard JA, Warwick G. 1993. Butyrate rapidly induces growth inhibition and differentiation in HT-29 cells. *Cell Growth Differ* 4: 495-501.
12. Frankel WL, Zhang W, Singh A, Klurfeld DM, Don S, Sakata T, Modlin I, Rombeau JL. 1994. Mediation of the trophic effects of short-chain fatty acids on the rat jejunum and colon. *Gastroenterology* 106: 375-380.
13. Coconnier MH, Bernet-Camard MF, Servin AL. 1994. How intestinal epithelial cell differentiation inhibits the cell-entry of Yersinia pseudotuberculosis in colon carcinoma Caco-2 cell line in culture. *Differentiation* 58: 87-94.
14. Huang N, Katz JP, Martin DR, Wu GD. 1997. Inhibition of IL-8 gene expression in Caco-2 cells by compounds which induce histone hyperacetylation. *Cytokine* 9: 27-36.
15. Andoh A, Fujiyama Y, Hata K, Araki Y, Takaya H, Shimada M, Bamba T. 1999. Counter-regulatory effect of sodium butyrate on tumuor necrosis factor-alpha (TNF-α)-induced

complement C3 and factor B biosynthesis in human intestinal epithelial cells. *Clin Exp Immunol* 118: 23-29.
16. Ren H, Musch MW, Kojima K, Boone D, Ma A. 2001. Short-chain fatty acids induce intestinal epithelial heat shock protein 25 expression in rats and IEC 18 cells. *Gastroenterology* 121: 631-639.
17. Fusunyan RD, Quinn JJ, Fujimoto M, MacDermott RP, Sanderson IR. 1999. Butyrate switches the pattern of chemokine secretion by intestinal epithelial cells through histone acetylation. *Mol Med* 5: 631-640.
18. Fusunyan RD, Quinn JJ, Ohno Y, MacDermott RP, Sanderson IR. 1998. Butyrate enhances interleukin (IL)-8 secretion by intestinal epithelial cells in response to IL-1β and lipopolysaccharide. *Pediatr Res* 43: 84-90.
19. Sanderson IR. 1997. Diet and gut inflammation. *Curr Opin Gastroenterol* 13: 518-524.
20. Scheppach W, Sommer H, Kirchner T, Paganelli GM, Bartram P, Christl S, Richter F, Dusel G, Kasper H. 1992. Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. Gastroenterology 103: 51-56.
21. Musch MW, Sugi K, Straus D, Chang EB. 1999. Heat shock protein 72 protects against oxidant-induced injury of barrier function of human colonic epithelial Caco-2/bbe cells. *Gastroenterology* 117: 115-122.
22. Liang P, MacRae TH. 1997. Molecular chaperones and the cytoskeleton. *J Cell Sci* 110: 1431-1440.
23. Koyasu S, Nishida E, Kadowaki T, Matsuzaki F, Iida K, Harada F, Kasuga M, Sakai H, Yahara I. 1986. Two mammalian heat shock proteins, HSP90 and HSP100, are actin binding proteins. *Proc Natl Acad Sci USA* 83: 8054-8058.
24. Mackie RI, Sghir A, Gaskins HR. 1999. Developmental microbial ecology of the neonatal gastrointestinal tract. *Am J Clin Nutr* 69: 1035S-1045S.
25. Nurmi E, Rantale M. 1973. New aspects of *Salmonella* infection in broiler production. *Nature* 241: 210-211.
26. Lloyd AB, Cumming RB, Kent RD. 1974. Presented at the proceedings of the Australian poultry science convention, Hobart, Tasmania.

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THE HEAT SHOCK RESPONSE AND CYTOPROTECTION OF THE INTESTINAL EPITHELIUM

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Abstract

Following heat stress, mammalian intestinal epithelial cells respond by producing heat shock proteins that confer protection under stressful conditions, which would otherwise lead to cell damage or death. Some of the noxious processes against which the heat shock response protects cells include heat stress, infection, and inflammation. The mechanisms of heat shock response-induced cytoprotection involve inhibition of proinflammatory cytokine production and induction of cellular proliferation for restitution of the damaged epithelium. This can mean selective interference of pathways, such as nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK), that mediate cytokine production and growth responses. Insight into elucidating the exact protective mechanisms could have therapeutic significance in treating intestinal inflammations and in aiding maintenance of intestinal integrity. Herein we review findings on heat shock response-induced intestinal epithelial protection involving regulation of NF-κB and MAPK cytokine production.

Introduction

The intestinal epithelium is exposed to an array of injurious agents ranging from pathogens like viruses or bacteria to their products, xenobiotics, chemicals, immune and inflammatory cytokines, and thermal and related stress stimuli. To some extent, it serves as a protective barrier between these agents and the sterile host environment. Exposure to such noxious stimuli may lead to a complex, but well-coordinated, signal transduction process to maintain intestinal integrity and function. The well-coordinated mechanisms result in increased proliferation of crypt cells, secretion of enzymes, and synthesis and secretion of immune and inflammatory cytokines and heat shock proteins (Hsps).

Following inflammation inducing stimuli, such as pathogens or proinflammatory cytokines, a transcriptional activator of several genes, nuclear factor kappa B (NF-κB), is activated[1]. Concurrently, the mitogen-activated protein kinase (MAPK) pathway can be activated. The activation leads to the expression of cytokine receptors, cell adhesion molecules, viral genes, and various inflammatory cytokines, including neutrophil chemoattractants that attract leukocytes to the respective sites to induce inflammation[2-7]. In addition, MAPK is activated by stress and growth factors that modulate the transcription of genes coding for protective and growth proteins leading to cellular proliferation and migration that are vital for restitution of the damaged epithelium. NF-κB is a critical regulator of the early pathogen response and an activator of the immune mediators. On the other hand, thermal stress induces the production of the putative Hsps through activation of heat shock transcription factor (HSF). The Hsps produced protect cells against further injury by rescuing intracellular proteins from irreversible denaturation; hence the term 'chaperones'[8]. Two groups of proteins, Hsps and proinflammatory cytokines, seem to operate antagonistically. Interestingly, anti-inflammatory cytokines that oppose the proinflammatory cytokines seem to work in favor of the Hsps for cytoprotection. Accumulating evidence reveals that Hsps suppress inflammatory gene expression and thereby inhibit the synthesis of inflammatory cytokines to curb inflammation. Blockade of NF-κB or MAPK-mediated inflammatory responses by Hsps or other agents can be of therapeutic significance. However, the actual mechanisms by which Hsps may act to suppress inflammatory cytokine production through these pathways are incompletely understood.

Because of the emerging significance of cytoprotection by intracellular mediators, we decided to review the possible roles of Hsps in regulating inflammatory pathways that may be significant for intestinal protection.

Proinflammatory cytokine production

The NF-κB pathway

Although production of inflammatory cytokines in the intestinal mucosa is mainly a function of specialized cells of the immune system, such as the intraepithelial lymphocytes and other monocytes, the intestinal epithelial cells (IEC) are also involved in the intestinal defense. They are known to produce an array of inflammatory cytokines either constitutively or after stimulation by pathogens such as viruses and bacteria, proinflammatory cytokines, ionization radiation, and chemicals such as phorbol myristate acetate (PMA) [9,10]. Most, if not all, of the produced inflammatory cytokines are mediated by the transcriptional activator NF-κB through the NF-κB pathway[11] (Fig 2.1). The NF-κB is a p50-p65 Rel family protein heterodimer that transcribes various genes. The Rel family proteins are composed of 2 groups. One group consists of p50 (NF-κB1) and p52 (NF-κB2). This group has deoxyribonucleic acid (DNA)-binding and dimerization domains and a nuclear localization signal. The second group consists of p65 (Rel A), Rel (c-Rel), and Rel B. In addition to DNA-binding and dimerization domains, the second group is composed of transcriptional activation domains[9]. The NF-κB normally occurs in its inactive form bound to the inhibitory kappa B (IκB) family proteins (IκB-α, IκB-β, IκB-γ, and BcI-3) in the cytoplasm. Activation of the transcriptional activity of the NF-κB requires the phosphorylation of IκB proteins and their subsequent degradation to generate the p50-p65 that translocates into the nucleus and activates the respective genes[9].

From the NF-κB-dependent cytokine production pathway, it can be deduced that blockade of this pathway at any point to inhibit its transcriptional activation reduces or arrests the production of the inflammatory cytokines and hence inflammation. Anti-inflammatory cytokines such as interleukin (IL)-10 and IL-4, nonvirulent bacteria such as *Salmonella* spp, intestinal bacterial fermentation products like short-chain fatty acids, and the heat shock response repress inflammatory cytokine production by abrogation of some steps in the NF-κB pathway[12-15].

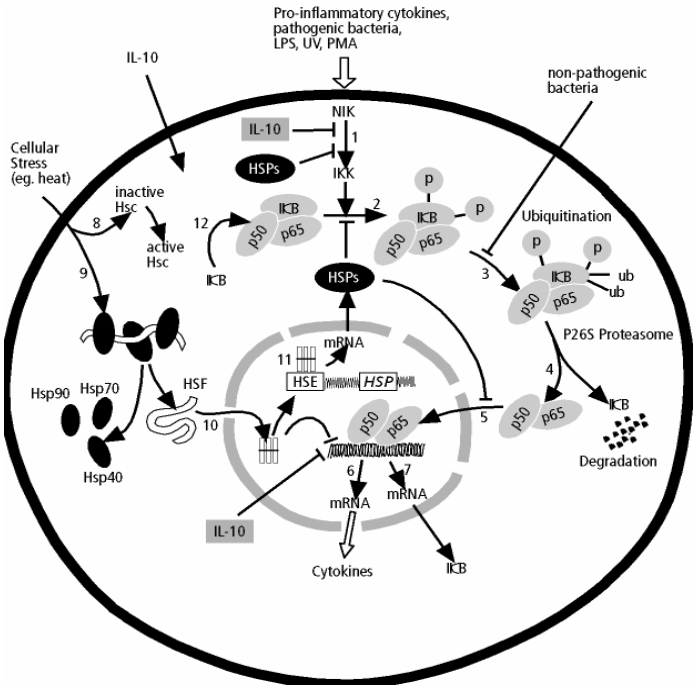

Fig 2.1. Cytokine regulation by NFκB pathway and the interaction with stress response. *Inflammatory agents activate NIK that in turn activates IKK (1) to phosphorylate IκB (2). Phosphorylated IκB is ubiquitinated (3) prior to proteasome degradation of IκB (4) that releases free p50-p65. The heterodimer p50-p65 translocates into the nucleus (5) for transcriptional activation of inflammatory cytokines (6) and IκB (7). Stress stimuli acts on constitutive HSPs (8) and activates cytoplasmic bound inactive monomeric HSF (9) by freeing binding Hsps. HSF then translocates into the nucleus (10), trimerises and activates HSE to produce HSPs (11). Both inducible and activated constitutive HSPs may suppress cytokine production by inhibiting activation of IKK (1), stabilizing NFκB/IκB complex (2), or maintaining p65 in the cytoplasm (5). The HSF may repress expression of inflammatory cytokines (6). Anti-inflammatory cytokines may inhibit IKK activation (1) or inflammatory gene expression (6). Over-expression of IκB stabilizes the NFκB/IκB complex (12) to inhibit degradation. Non-pathogenic bacteria abrogate ubiquitination (3).*

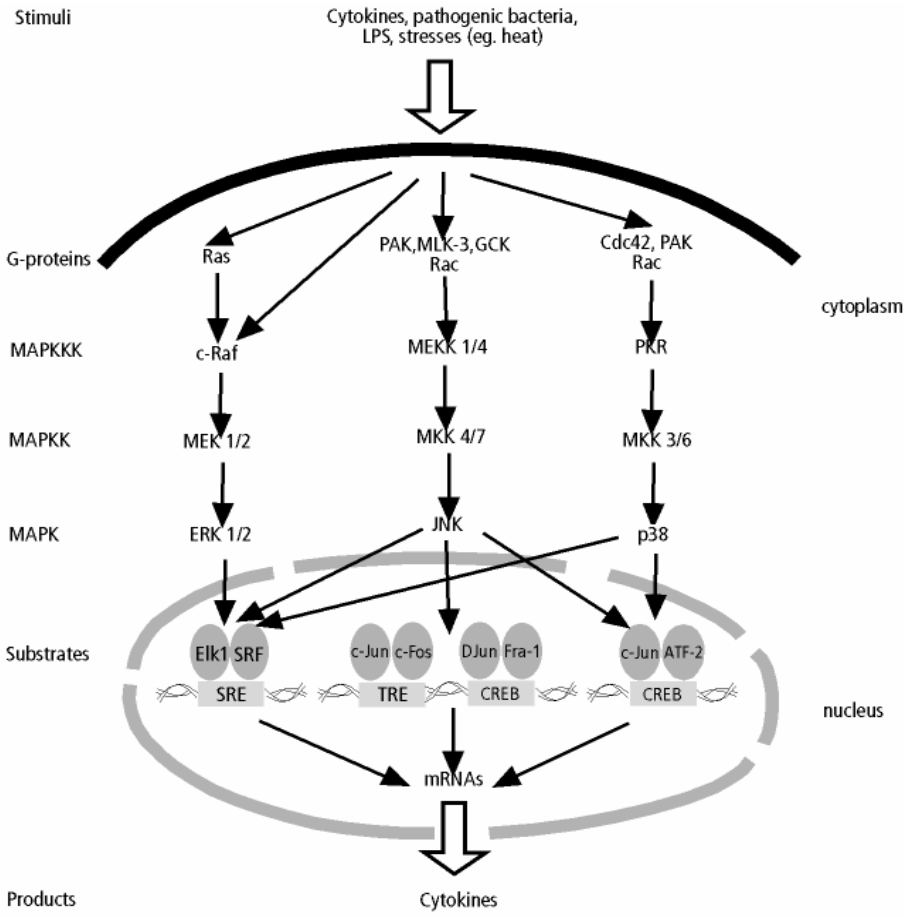

Fig 2.2. Cytokine production by MAPK pathways. *Various inflammatory agents and stress stimuli activate MAPK pathways and their subsequent substrates leading to transcriptional activation of genes coding for cytokines. This activation is followed by production of an array of inflammatory cytokines. See text for details and Table 1 for summarized specific pathway stimulation and respective cytokine production.*

The MAPK pathways

Some cytokine secretions are signaled through the MAPK pathways that are known to transduce the extracellular stress signals. These pathways consist of extracellular signal-regulated kinases (ERK) 1/2, c-Jun N-terminal kinases (JNK) (also known as stress- activated protein kinases), and p38. Their activation is through cascades of MAPK, MAPK kinases, and MAPK kinase kinases that are in turn activated by the various extracellular stimuli (Fig 2.2). Inflammatory cytokines, such as IL-1β, IL-17, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ, pathogenic bacteria, such as *Salmonella typhimurium* and *Escherichia coli*, lipopolysaccharides (LPS), thermal and oxidative stresses, PMA, and growth responses can all activate the MAPK-signaling cascades in the IEC, leading to the activation of genes coding for inflammatory cytokines[3-7,16]. Some of the cytokines mediated through MAPK in IEC are listed in Table 2.1.

Table 2.1 Cytokine production by IEC in association with activated MAPK pathways

Inducing agent	MAPK pathway	Function	Reference
IL-1β, IL–17, TNF-α	Ras-mediated JNK, p38, ERK1/2	Coactivation of NF-κB that produces CINC, MCP-1	Awane et al ,1999
IL-10	JNK	Early protection	Zingarelli et al, 2001
Absence of IL-10	Augmented JNK	Production of IL-6, TNF-α	Zingarelli et al, 2001
IFN-γ	Fas-mediated JNK	Production of IL-8	Martin et al, 1999
TGF-β	Ras-mediated ERK1/2, JNK	Production of TGF-β_1	Yue and Mulder, 2000
S. typhimurium	ERK1/2, JNK, p38	Production of IL-8	Hobbie et al, 1997
EPEC	ERK1/2, JNK, p38	Production of IL-8	Czerucka et al, 2001
Stxs	P38	Production of IL-8	Thorpe et al, 1999
IL-1β	JNK	Production of IL-6	Hungness et al, 2000
LPS	ERK1/2, JNK, p38	Coactivation of NF-κB	Cario et al, 2000

EPEC, enteropathogenic *Escherichia coli;* Stxs, *E. coli* Shiga toxins; CINC, cytokine-induced neutrophil chemoattractant; MCP-1, monocyte chemoattractant protein-1; PMA, phorbol myristate acetate; IEC, intestinal epithelial cells; MAPK, mitogen-activated protein kinase; IL, interleukin; TNF, tumor necrosis factor; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; NF-κB, nuclear factor-κB; IFN, interferon; TGF, transforming growth factor; LPS, lipopolysaccharide.

The role of the heat shock response in cytokine regulation

The heat shock response and its protection of the IEC

Most cells and organisms react to heat and a variety of stressors by rapid synthesis of a group of evolutionary conserved proteins, ranging in size from 8 kDa to 150 kDa[8,17], termed Hsps. They are classified into several families according to their molecular weights. The major Hsp families include HSP150, HSP110, HSP90, HSP70, HSP60, HSP40, HSP20, and HSP8.5 (Table 2).

The heat shock response is induced by various stimuli, including thermal stress, heavy metals such as sodium arsenite and zinc ions, bacteria, and bacterial exo- and endotoxins, viral infections, ischemia, nutritional deficiency, ionising radiation, oxidants, some IFN inducers, and cytokines in different cells including IEC[8]. Following heat shock or other stresses, Hsps are produced after transactivation of the genes by a family of DNA-binding proteins called the HSFs (HSF1-4, of which the best known is HSF1). In unstressed cells the inactive HSF is bound to the cytoplasmic Hsp40 (Hdj-1), Hsp70, and Hsp90 in a monomeric form without the DNA-binding activity. In response to stress, HSF is released and translocated into the nucleus, where it assembles into a trimer and binds to a specific consensus heat shock regulatory element (HSE) in the heat shock gene promoter to exert the transcriptional activation[18,19] (Fig 2.3). Heat shock response also activates constitutive Hsps that, together with the induced Hsps, affect cytoprotection.

The various Hsp families are differentially expressed in the IEC and may relate to the type or intensity of the epithelial damage, or location of the IEC. Hsp60, Hsp72, and Hsp90 are expressed in the colonic mucosa after hyperthermia. In response to acetic acid-induced intestinal lesions, Hsp72 and Hsp90 inductions are protective. Their inductions precede that of Hsp60, which has no protective effect[20]. Although Hsp72 is induced by cellular injury, intestinal motility may be enough to induce Hsp60. Kuwabara et al (1994) and Sasahara et al (1998) demonstrated that water immersion stress that causes functional diarrhea without histopathological changes induces Hsp60 but not Hsp72 nor Hsp90 in both colon and small IEC[21,22]. In these cases Hsp60 was observed to have no protective role against acetic acid-induced intestinal lesions. Hsp72 and Hsp73 have also been reported to have no protective function against small IEC indomethacin-induced injuries[23]. The chaperone function for a particular Hsp may, therefore, be specific to certain intestinal injuries or type and location of the IEC along the alimentary tract.

Table 2.2 Major heat shock proteins (Hsps) known in mammalian cells

Family	Hsps	Function
HSP150	Hsp150	Molecular chaperone in ischemia and hypoxia
HSP110	Hsp110	Molecular chaperone in hyperthermia and ischemia
	Hsp105	Molecular chaperone in hyperthermia and ischemia
HSP90	Hsp100	Molecular chaperone for secretory proteins
	Hsp90	Molecular chaperone in hyperthermia and ischemia and for protein kinases. Translocation of cytosolic TNF-α and Raf kinases to plasma membrane. Steroid hormone receptor functions. Cell proliferation and growth
HSP70	Grp78	Molecular chaperone in hyperthermia. Protein trafficking across endoplasmic reticulum
	Hsp72	Molecular chaperone in hyperthermia, hypoxia, and ischemia. Inhibits leukotriene production
	Hsc70	Constitutive molecular chaperone for hyperthermia protection.
	Hsp70	Highly induced molecular chaperone in hyperthermia and ischemia. Regulates the heat shock response
	MtHsp70	Mitochondrial Hsp70 molecular chaperone
HSP60	Hsp60	Molecular chaperone (chaperonin) in hyperthermia and ischemia
	Hsp65	Growth stress responses for tumor regression
	TCP-1	Molecular chaperone (chaperonin) in hyperthermia and ischemia
HSP40	Hsp47	Molecular chaperone for collagen
	Hsp40	Hsp70 cofactor for mediation of most Hsp70 functions
HSP20	Hsp27	Molecular chaperone in hyperthermia, chemicals (hydrogen peroxide, drugs), and irradiation. Actin protection. Enhances IL-10 production
	Hsp20	Molecular chaperone in hyperthermia, ischemia, and hydrogen peroxide

Grp, glucose-regulated protein; Hsc70, constitutive Hsp70; mtHsp70, mitochondrial Hsp70; TCP-1, T-complex protein; TNF, tumor necrosis factor; IL, interleukin.

Cytokines involved in heat shock induction imply an interrelationship among these mediators. Because of their protective role, Hsps can be expected to down-regulate inflammatory cytokines to overcome inflammation. This suggests a mechanism of modulating cytokine production by both NF-κB and MAPK pathways. Several studies have shown that the heat shock response does, in fact, inhibit some cytokine production mediated by NF-κB and modulates MAPK-dependent cytokine production in a specific manner.

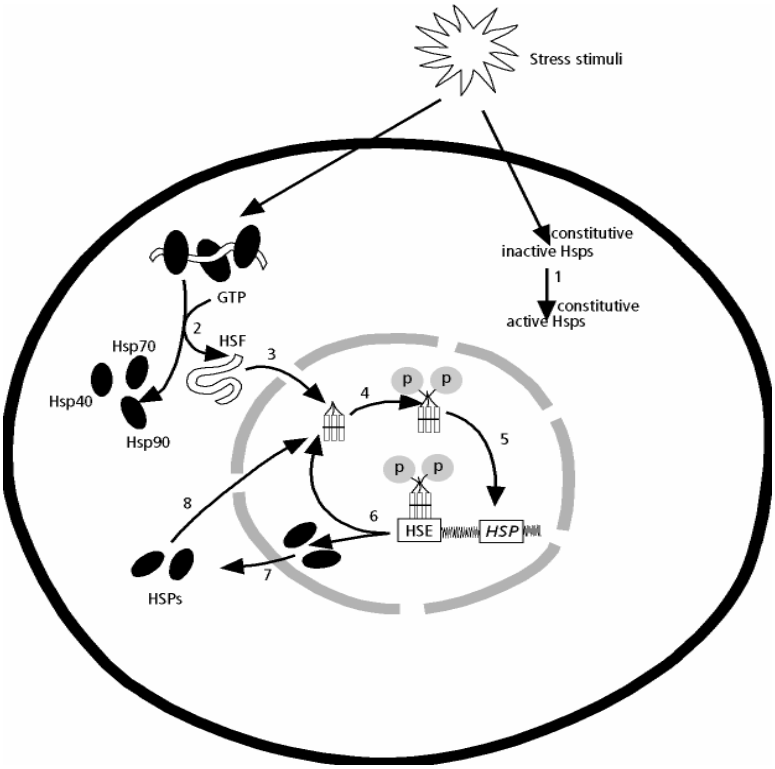

Fig 2.3. Regulation of HSPs. *Stress stimuli activates inactive form of constitutive HSPs into active form (1). The inactive monomer non-DNA binding cytoplasmic HSF that resides bound to HSPs in unstressed cells is activated by stress stimuli and dissociates into HSPs and DNA-binding HSF (2). The active HSF translocates into the nucleus (3), trimerises and binds to HSPs gene promoter prior to undergoing phosphorylation at serine residues (4). Phosphorylated HSF attaches to the HSE located upstream of the HSPs genes (5) followed by transcription activation that results into production of HSPs and HSF (6). The HSF produced maintains the circle while HSPs are released into cytoplasm (7). High levels of cytoplasmic HSPs causes nuclear localization of HSPs (8) that in turn, bind to HSF to repress HSPs transcriptional activation.*

Hsps inhibit the NF-κB inflammatory cytokine production by IEC

There is accumulating evidence to suggest that Hsp induction abrogates the activation of the NF-κB inflammatory pathway and thereby inhibits proinflammatory gene expression[24,25]. Hsps appear to inhibit NF-κB transcriptional activation either by inhibiting IκB degradation or by directly repressing the NF-κB transcriptional activity[25-27]. Hsps are believed to prevent IκB degradation by inhibiting IκB kinases (IKK) activation, but how this occurs is unclear.

The normally occurring cytoplasmic IκB-NF-κB complex exists via an interaction between the IκB-α ankyrin domains and the nuclear localization sites. Human HSP70 has nuclear localization sites[28]. The presence of these nuclear localization sites raises the possibility that Hsps can specifically interact with the consensus IκB-α ankyrin domain and in turn hamper IκB-NF-κB phosphorylation and the subsequent IκB degradation[27].

There is strong evidence that Hsps enhance IκB production. In this case, elevated levels of IκB stabilize the IκB-NF-κB complex, resulting in hampered IκB degradation. Wong et al (1997) identified a 20-bp heat shock responsive segment in the human IκB-α that could be a functional heat shock responsive element in NF-κB transcriptional inhibition[29]. In their study stress induced IκB-α messenger ribonucleic acid (mRNA) and protein expression, stabilizing the IκB-NF-κB association and suppressing NF-κB trascriptional activation. Consistently, Pritts et al (2000) observed that heat stress was associated with the maintained IEC cytoplasmic IκB-α levels and the decreased endotoxin-induced NF-κB DNA-binding transcriptional activity[30]. Both studies suggest dual mechanisms for NF-κB inhibition by heat shock response, increased expression of IκB-α and inhibition of the degradation of IκB-α.

Chaperones function by shielding the already synthesized proteins from degradation, mediating these stabilizing effects through protein-protein interaction. Stabilization of IκB-α against phosphorylation and degradation following stress responses could partly be through this mechanism as well.

Though the mechanism is enigmatic, HSF acts as a transcriptional repressor of the cytokine genes. Cahill et al (1996) in their study on human monocytes showed that HSF represses the IL-1β gene responding to LPS by binding to a specific HSE in the IL-1β promoter[24]. They suggested transcriptional repressor mechanisms that were distinct from those involved in the activation. In turn, this could block other cytokines that are secreted in response to intracellular IL-1β. This offers another potential mechanism for the down-regulation of cytokine expression by Hsps in IEC.

Hsps modulate the MAPK pathway to confer IEC protection

The protective role of Hsps in the IEC occurs through the modulation of the MAPK pathways. Hsps may selectively influence ERK1/2, JNK and p38 MAPK pathways in various stressful conditions[31-34]. Though most Hsps signal through the Ras-Raf-independent ERK1/2 MAPK pathway, the activation of the down-stream genes may be specific. Hsp90 mediates normal IEC growth signals via ERK1/2, thereby protecting cells against apoptosis[35]. Likewise, sodium arsenite induces Hsp70 synthesis in IEC via ERK1/2 activation of the HSF[36]. This Hsp70 together with Hsc70 suppress JNK signaling, leading to cellular protection against various stresses[37]. The JNK pathway has a potential to down-regulate IL-10 production to favor intestinal inflammation. Hence, its suppression by Hsp70 and Hsc70 is vital for anti-inflammatory responses. Interestingly, Hsp27 and Hsp72 prevent both repression of IL-10 production and induction of apoptosis by modulating the activity of the JNK. Subsequently, the accumulated Hsps suppress JNK to protect cells against stresses[32,33].

IL-6 production by the IEC after AP-1 activation by stress response is mediated through the JNK pathway. The stimulated JNK pathway activates c-Jun and c-Fos heterodimer members of AP-1 leading to IL-6 production[38,39]. Though IL-6 is a proinflammatory cytokine, its production after heat shock induction could be protective. Barton and Jackson (1993) demonstrated a protective role for IL-6 against death from septic shock in mice[40]. In their study IL-6 was observed to confer a significant reduction of the LPS-induced septic shock mortality.

Cross talk between HSF, NF-κB and MAPK signal transduction pathways

Cellular responses at the gene level are highly conserved. The HSFs responding to establish cytoprotection after the heat shock response, the NF-κB for inducing inflammation and the MAPK responding to both protection and inflammation, seem to be coordinated in a very specific way. In this coordination, the activation of the HSFs antagonizes the inflammatory activities of MAPK and NF-κB while favoring the anti-inflammatory responses. Similarly, inflammatory responses mediated by both MAPK and NF-κB seem to complement, if not support, each other.

Inhibition of the activities of the inflammatory cytokine TNF-α in the IEC is associated with the inhibition of the NF-κB and MAPK inflammatory responses. Interestingly, this modulation favors the protective, proliferative MAPK responses,

induced by the epidermal growth factor, that are important in epithelial restitution after injury[41].

In other systems, an increase in the HSF DNA-binding activity and the subsequent Hsp production is associated with the enhanced protective influences of the ERK1/2 MAPK pathway and the suppressed JNK and p38 MAPK responses. Consistently, a decrease in the Hsp production is accompanied by an increase in the JNK MAPK activity that favors inflammatory responses. Absence of the protective ERK1/2 MAPK pathway blocks HSF transcription and Hsp up-regulation[42,43]. Hsp protection of the IEC through the inhibition of JNK activation is also reported. Sodium arsenite induces Hsp70 in the IEC via the ERK1/2 MAPK pathway. This induction is associated with the HSF activation. Hsp70 together with Hsc70 produced through this pathway suppress the JNK pathway to confer cytoprotection[37].

The high constitutive levels of Hsp90 observed in unstressed cells[44] seem to play a vital role in modulating cellular protection through its interaction with other Hsps and various kinases. Recently, Hsp90 was found to be a repressor of the double-stranded ribonucleic acid-dependent protein kinase PKR, and its inhibition activates the kinase[45]. PKR is activated in response to viral infection, and it favors inflammation by interacting with IKK to catalyse NF-κB transcriptional activation[46,47]. In addition, PKR activates MAPK p38 and contributes to LPS-induced IL-6 and IL-12 production[48]. These findings indicate a pivotal role of PKR linking NF-κB and MAPK that is controlled by Hsps. The phenomenon is such that the constitutive levels of Hsp90 suppress the PKR and thereby keep NF-κB inactive. Blockade of this Hsp not only activates PKR but also enhances the p38 activity and the ubiquitin-dependent proteasome degradation, a mechanism important for NF-κB activation[49].

The inhibition of Hsp production may also account for the decreased Hsp-augmented IEC production of IL-6 that is partly associated with the inhibition of the AP-1 activation[50]. Inhibiting AP-1 activation suppresses inflammatory cytokine production even without inactivating NF-κB, indicating that blockade of either MAPK or NF-κB decreases inflammatory cytokine production. The production of both IL-8 and IL-6 depends on both pathways, and the inhibition of p38 MAPK and subsequent AP-1 activation abolish their production[3,50]. Interestingly, inhibiting NF-κB ubiquitin-dependent proteasome degradation by proteasome inhibitors activates HSF and AP-1 through p38 and JNK MAPK pathways[51], resulting in a protective role. This implies a vital role for constitutive Hsps with regard to mediating signals to both MAPK and NF-κB pathways (Fig 2.4).

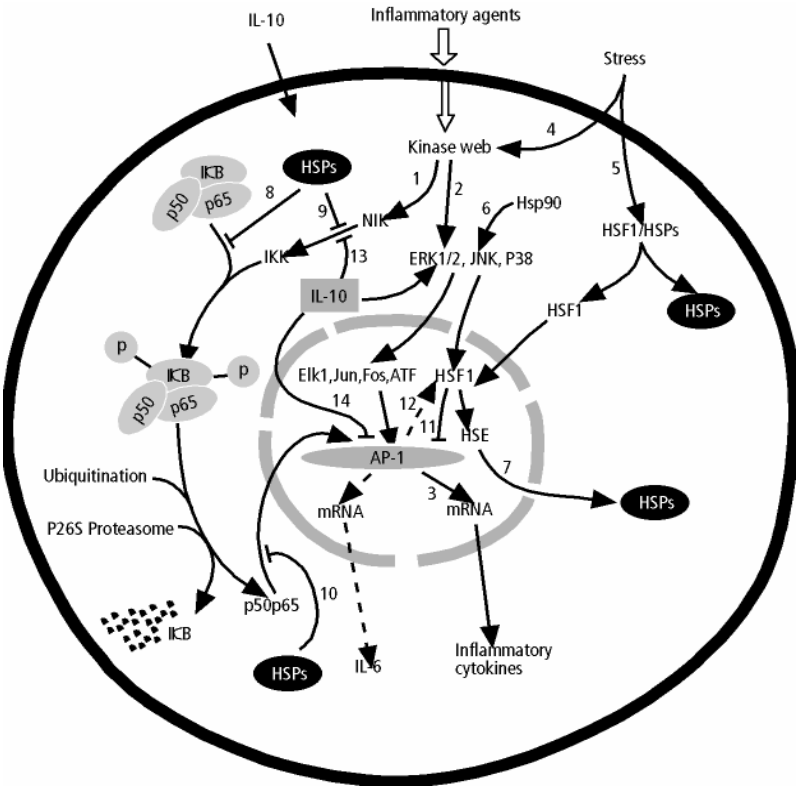

Fig 2.4. Interaction of stress and inflammatory responses. *Inflammatory agents activate NF-κB (1) and MAPK pathways (2) through several kinases resulting into activation of AP-1 and subsequent production of inflammatory cyokines (3). Stress factors activate MAPK pathway through a series of kinases (4) and HSF1 (5) that translocates into the nucleus. The MAPK pathway is also activated by constitutive Hsp90 (6). Activated HSF1 binds to HSE followed by HSPs production (7) that block NF-κB pathway (8, 9, 10). The HSF1 may inhibit AP-1 inflammatory cytokine transcription activation (11). AP-1 has potential to activate HSF1 transcriptional activation (12). IL-10 blocks both IKK activation (13) and AP-1 activation (14) to repress inflammatory cytokine production. Dotted arrows represent stress response AP-1 products that are not produced by NF-κB. For more details see text.*

These observations imply a tightly regulated communication network among various signal transduction pathways to elicit cellular and stimuli specific responses. HSF transcription and Hsp up-regulation is associated with protective MAPK activities mainly mediated through the ERK1/2 pathway. On the other hand, NF-κB inflammatory responses are connected with JNK and p38 MAPK pathways. More importantly, all 3 MAPK pathways can be activated at one time, but their responses are highly insulated from one another.

Therapeutic significance

Selective blockade of the inflammatory NF-κB and MAPK pathways is important in reducing intestinal inflammations. Manipulations of these pathways are underway as an effective approach to inhibit proinflammatory gene expression. The principle mechanisms may be to activate HSF and MAPK protective responses while inhibiting inflammatory NF-κB and MAPK pathways.

A number of studies suggest a close reciprocal relationship between the activities of HSF and NF-κB. In these studies the inhibitors of NF-κB activation that effectively prevented IκB-α degradation were potent activators of HSF transcription. Prostaglandin A and J were found to activate HSF and induce the synthesis of Hsps that protected cells against hyperthermia and virus infection[52]. These prostaglandins were potent inhibitors of NF-κB transcription activation[53]. Though their dual effects may neither be dependent nor be linked to one another, these findings strengthen the approach that maneuvering either of the pathways could be of therapeutic significance. Javadpour et al (1998) observed that a tyrosine kinase inhibitor herbimycin-A protected ischemic animals by inhibiting neutrophil infiltration[54], a sequel of the NF-κB-dependent chemokine synthesis. They suggested that the effect was because of the increased expression of Hsps by activated HSF.

The observations that enhanced ERK1/2 and suppressed JNK and p38 in association with up-regulation of Hsps confer protection against stress seem to be the principle underlying the working of some drugs. In such cases the absence of ERK1/2 that subsequently abolishes HSF transcriptional activation may render the drugs ineffective[42,43]. By contrast, the activation of ERK1/2 by enteropathogenic *E. coli* via phosphorylation of the upstream Hsp54 enhances bacterial internalization[55]. Under such circumstances, prevention of phosphorylation of such Hsps may have therapeutic importance.

Advances in the direct inhibition of the inflammatory NF-κB or MAPK pathway without the involvement of Hsps are well reported. Potent inflammatory

bowel disease drugs, mesalamine (5-aminosalicylic acid) derivatives, inhibit both inflammatory pathways[41]. Cytokine-suppressive anti-inflammatory drugs block the production of proinflammatory cytokines at a posttranslational level by binding to p38 and subsequently inhibiting its kinase activity[56]. Working together with NF-κB activation for cytokine production, inhibition of p38 could overcome both NF-κB and MAPK inflammatory responses[3]. Similarly, intracolonic introduction of single-stranded molecules of 15-25 bp (antisense phosphorothioate oligonucleotides) that can hybridize to the p65 mRNA and inhibit its expression has been shown to decrease the transcriptional activation of NF-κB. This medication lowers the synthesis of the proinflammatory cytokines IL-1, IL-6, and TNF-α and improves intestinal inflammation[57].

Although inhibition of proinflammatory cytokines by the blockade of the NF-κB or MAPK pathway aids curb inflammation, long-term suppression of these pathways in humans or animals may not be a good idea to advocate for treating intestinal inflammations. The reasons partly stem from the findings by Inan et al (2000) and Erdman et al (2001) who showed high cytokine expression by the IECs of knockout and heterozygote mouse strains with less NF-κB[58,59]. This may imply that NF-κB plays an important role in maintaining intestinal homeostasis and that other transcription factors can play a major role in inflammatory gene expression. In other cell systems prolonged inhibition of NF-κB results in massive cell death by apoptosis[60].

Conclusions

The maintenance of the normal intestinal function at all situations is complex and involves many factors. These factors are regulated at multiple levels and interact with one another in a highly organized manner. Although NF-κB and MAPK pathways are involved in the production of inflammatory cytokines and cellular proliferation, their modulation by the heat shock response is beneficial for diverse harmful stimuli. Generally, the resulting Hsps not only protect cells by acting as chaperones but also down-regulate proinflammatory cytokine production to curb noxious processes, like heat stress, infection, and inflammation. In reality, the resulting heat shock response-induced cytoprotection may culminate in the restoration of the intestinal epithelial function.

References

1. Rogler G, Brand K, Vogl D, Page S, Hofmeister R, Andus T, Knuechel R, Baeuerle PA, Scholmerich J, Gross V. 1998. Nuclear factor κB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. *Gastroenterology* 115: 357-369.
2. Baldwin A. 1996. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* 14: 649-683.
3. Hobbie S, Chen LM, Davis RJ, Galan JE. 1997. Involvement of mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by *Salmonella typhimurium* in cultured intestinal epithelial cells. *J Immunol* 159: 5550-5559.
4. Awane M, Andres PG, Li DJ, Reinecker HC. 1999. NF-κB-inducing kinase is a common mediator of IL-17, TNF-α-, and IL-1β-induced chemokine promoter activation in intestinal epithelial cells. *J Immunol* 162: 5337-5344.
5. Martin A, Maria T, Andrew A, Palladino AA, Faris M, Carramanzana NM, Nel AE, Targan SR. 1999. Fas activates the JNK pathway in human colonic epithelial cells: lack of a direct role in apoptosis. *Am J Physiol* 276: G599-G605.
6. Cario E, Rosenberg M, Brandwein SL, Beck PL, Reinecker HC, Podolsky DK. 2000. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *J Immunol* 164: 966-972.
7. Yue J, Mulder KM. 2000. Requirement of Ras/MAPK pathway activation by transforming growth factor β_1 production in a smad-dependent pathway. *J Biol Chem* 275: 30765-30773.
8. Wu C. 1995. Heat shock transcription factors: Structure and regulation. *Annu Rev Cell Dev Biol* 11: 441-469.
9. Thanos D, Maniatis T. 1995. NF-κB: a lesson in family values. *Cell* 80: 529-532.
10. Elewaut D, DiDonato JA, Kim JM, Truong F, Eckmann L, Kagnoff MF. 1999. NF-κB is a central regulator of the intestinal epithelial cell innate immune response induced by infection with enteroinvasive bacteria. *J Immunol* 163: 1457-1466.
11. Baeuerle PA, Henkel T. 1994. Function and activation of NF-κB in the immune system. *Ann Rev Immunol* 12: 141-179.
12. Schottelius AJG, Mayo MW, Sartor RB, Bardwin AS, Jr. 1999. Interleukin-10 signaling blocks inhibitor of κB kinase activity and nuclear factor κB DNA binding. *J Biol Chem* 274: 31868-31874.
13. Wu GD, Huang N, Wen X, Keilbaugh SA, Yang HJ. 1999. High-level expression of IκB-β in the surface epithelium of the colon: *in vitro* evidence for an immunomodulatory role. *J Leukoc Biol* 66: 1049-1056.
14. Neish AS, Gewirtz AT, Zeng H, Young AN, Hobert ME, Karmali V, Rao AS, Madara JL. 2000. Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination. *Science* 289: 1560-1563.
15. Berin MC, Dwineli MB, Eckmann L, Kagnoff MF. 2001. Production of MDC/CCL22 by human intestinal epithelial cells. *Am J Physiol* 280: G1217-G1226.
16. Czerucka D, Dahan S, Mograbi B, Rossi B, Rampal P. 2001. Implication of mitogen-activated protein kinases in T84 cell responses to enteropathogenic *Eschelichia coli* infection. *Infect Immun* 69: 1298-1305.
17. Ovelgönne JH, Koninkx JFJG, Pusztai A, Bardocz S, Kok W, Ewen SWB, Hendriks HGCJM, van Dijk JE. 2000. Decreased level of heat shock proteins in gut epithelial cells after exposure to plant lectins. *Gut* 46: 679-687.
18. Santoro MG. 2000. Heat shock factors and the control of the stress response. *Biochem Pharmacol* 59: 55-63.

19. Han SI, Oh SY, Woo SH, Kim KH, Kim JH, Kim HD, Kang HS. 2001. Implication of a small GTPase Rac1 in the activation of c-Jun N-terminal kinase and heat shock factor in response to heat shock. *J Biol Chem* 276: 1889-1895.
20. Otani S, Otaka M, Jin M, Okuyama A, Itoh S, Iwabuchi A, Sasahara H, Itoh H, Tashima Y, Masamune O. 1997. Effect of preinduction of heat shock proteins on acetic acid-induced colitis in rats. *Dig Dis Sci* 42: 833-846.
21. Kuwabara T, Otaka M, Itoh H, Tashima Y, Zeniya A, Fujimori S, Masamune O. 1994. Regulation of 60-kDa heat shock protein expression by systemic stress and 5-hydroxytryptamine in rat colonic mucosa. *J Gastroenterol* 29: 721-726.
22. Sasahara H, Otaka M, Itoh S, Iwabuchi A, Odashima M, Wada I, Konishi N, Pacheco II, Tashima Y, Itoh H, Otani S, Masamune O. 1998. Effect of preinduction of heat-shock proteins on acetic acid-induced small intestinal lesions in rats. *Dig Dis Sci* 43: 2117-2130.
23. Jin M, Otaka M, Otani S, Okuyama A, Itoh S, Iwabuchi A, Sasahara H, Fujimori S, Itoh H, Tashima Y, Masamune O. 1997. Effect of pre-induction of heat shock proteins on indomethacin-induced small intestinal lesion in rats. *J Gastroenterol* 32: 34-39.
24. Cahill CM, Waterman WR, Xie Y, Auron PE, Calderwood SK. 1996. Transcriptional repression of the proinflammatory 1β gene by heat shock factor 1. *J Biol Chem* 271: 24874-24879.
25. Chu EK, Ribeiro SP, Slutsky AS. 1997. Heat stress increases survival rates in lipopolysaccharide-stimulated rats. *Crit Care Med* 25: 1727-1732.
26. Jobin C, Holt L, Bradham CA, Streetz K, Brenner DA, Sartor RB. 1999. TNF receptor-associated factor-2 is involved in both IL-1β and TNF-α signaling cascades leading to NF-κB activation and IL-8 expression in human intestinal epithelial cells. *J Immunol* 162: 4447-4454.
27. Yoo C, Lee S, Lee C, Kim YW, Han SK, Shim Y. 2000. Anti-inflammatory effect of heat shock protein induction is related to stabilization of IκBα through preventing IκB kinase activation in respiratory epithelial cells. *J Immunol* 164: 5416-5423.
28. Dang CV, Lee WMF. 1989. Nuclear and nucleolar targeting sequences of c-*erb*-A, c-*myb*, N-*myc*, p53, Hsp70, and HIV tat proteins. *J Biol Chem* 254: 18019-18023.
29. Wong HR, Ryan M, Wispé JR. 1997. Stress response decrease NF-κB nuclear translocation and increases I-κBα expression in A549 cells. *J Clin Invest* 99: 2423-2428.
30. Pritts TA, Wang Q, Sun X, Moon R, Fischer DR, Fischer JE, Wong HR, Hasselgren PO. 2000. Induction of the stress response *in vivo* decreases nuclear factor-kappa B activity in jejunal mucosa of endotoxemic mice. *Arch Surg* 135: 860-866.
31. Hill CS, Treisman R. 1995. Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell* 80: 199-211.
32. Tilly BC, Gaestel M, Engel K, Edixhoven MJ, de Jonge HR. 1996. Hypo-osmotic cell swelling activates the p38 MAP kinase signaling cascade. FEBS lett 395: 133-136.
33. Gabai VL, Meriin AB, Yaglom JA, Volloch VZ, Sherman MY. 1998. Role of HSP70 in regulation of stress-kinase JNK: implications in apoptosis and aging. *FEBS Lett* 438: 1-4.
34. Ng DCH, Bogoyevitch MA. 2000. The mechanism of heat shock activation of ERK mitogen-activated protein kinases in the interleukin 3-dependent pro-B cell line BaF3. *J Biol Chem* 275: 40856-40866.
35. Hostein I, Robertson D, DiStefano F, Workman P, Clarke PA. 2001. Inhibition of signal transduction by the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin results in cytostasis and apoptosis. *Cancer Res* 61: 4003-4009.
36. Chen YC, Tsai SH, Shen SC, Lin JK, Lee WR. 2001. Alternative activation of extracellular signal-regulated protein kinases in curcumin and arsenite-induced Hsp70 gene expression in human colorectal carcinoma cells. *Eur J Cell Biol* 80: 213-221.

37. Mosser DD, Caron AW, Bourget L, Meriin AB, Sherman MY, Morimoto RI, Massie B. 2000. The chaperone function of Hsp70 is required for protection against stress-induced apoptosis. *Mol Cell Biol* 20: 7146-7159.
38. Andoh A, Fujiyama Y, Hata K, Araki Y, Takaya H, Shimada M, Bamba T. 1999. Counter-regulatory effect of sodium butyrate on tumor necrosis factor –alpha (TNF-α)-induced complement C3 and factor B biosynthesis in human intestinal epithelial cells. *Clin Exp Immunol* 118: 23-29.
39. Yeh KY, Yeh M, Glass J, Granger DN. 2000. Rapid activation of NFκB and AP-1 and target gene expression in postischemic rat intestine. *Gastroenterology* 118: 525-534.
40. Barton BE, Jackson JV. 1993. Protective role of interleukin-6 in the lipopolysaccharide-galactosamine septic shock model. *Infect Immun* 61: 1496-1499.
41. Kaiser GC, Yan F, Polk B. 1999. Mesalamine blocks tumor necrosis factor growth inhibition and nuclear factor κB activation in mouse colonocytes. *Gastroenterology* 116: 602-609.
42. Kim SH, Kim D, Jung GS, Um JH, Chung BS, Kang CD. 1999. Involvement of c-Jun NH_2-Terminal kinase pathway in different regulation of heat shock proteins by anticancer drugs. *Biochem Biophys Res Com* 262: 516-522.
43. Tsuji M, Inanami O, Kuwabara M. 2000. Neuroprotective effect of α-phenyl-N-*tert*-butylnitrone in gerbil hippocampus is mediated by the mitogen-activated protein kinase pathway and heat shock proteins. *Neurosci Lett* 282: 41-44.
44. Buchner J. 1999. Hsp90 & Co.-a holding for folding. *Trends Biochem Sci* 24: 136-141.
45. Donze O, Abbas-Terki T, Picard D. 2001. The Hsp90 chaperone complex is both a facilitator and a repressor of the dsRNA-dependent kinase PKR. *EMBO J* 20: 3771-3780.
46. Zamanian-Daryoush M, Mogensen TH, DiDonato JA, Williams BR. 2000. NF-kappaB activation by double-stranded-RNA-activated protein kinase (PKR) is mediated through NF-kappaB-inducing kinase and IkappaB kinase. *Mol Cell Biol* 20: 1278-1290.
47. Gil J, Rullas J, Garcia MA, Alcami J, Esteban M. 2001. The catalytic activity of dsRNA-dependent protein kinase, PKR, is required for NF-kappaB activation. *Oncogene* 20: 385-394.
48. Goh KC, deVeer MJ, Williams BRG. 2000. The protein kinase PKR is required for p38 MAPK activation and the innate immune response to bacterial endotoxin. *EMBO J* 19: 4292-4297.
49. Schulte TW, Won GA, Neckers LM. 1997. Geldanamycin-induced destabilization of Raf-1 involves the proteosome. *Biochem Biophys Res Com* 239: 655-659.
50. Hungness ES, Pritts TA, Luo G, Sun X, Penner CG, Hasselgren PO. 2000. The transcription factor activator protein-1 is activated and interleukin-6 production is increased in interleukin-1β-stimulated human enterocytes. *Shock* 14: 386-391.
51. Tacchini L, Dansi P, Matteucci E, Bernelli-Zazzera A, Desiderio MA. 2001. Influence of proteasome and redox state on heat shock-induced activation of stress kinases, AP-1 and HSF. *Biochim Biophys Acta* 1538: 76-89.
52. Santoro MG. 1997. Antiviral activity of cyclopentenone prostanoids. *Trends Microbiol* 5: 276-281.
53. Rossi A, Elia G, Santoro MG. 1997. Inhibition of nuclear factor kappa B by prostaglandin A1: an effect associated with heat shock transcription factor activation. *Proc Natl Acad Sci USA* 94: 746-75.
54. Javadpour M, Kelly CJ, Chen G, Bouchier-Hayes DJ. 1998. Herbimycin-A attenuates ischaemia-reperfusion induced pulmonary neutrophil infiltration. *Eur J Vasc Endovasc Surg* 16: 377-382.
55. Czerucka D, Dahan S, Mograbi B, Rossi B, Rampal P. 2000. *Saccharomyces boulardii* preserves the barrier function and modulates the signal transduction pathway induced in enteropathogenic *Eschelichia coli*-infected T84 cells. *Infect Immun* 68: 5998-6004.

56. Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW, et al. 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* 372: 739-746.
57. Murano M, Maemura K, Hirata I, Toshina K, Nishikawa T, Hamamoto N, Sasaki S, Saitoh O, Katsu K. 2000. Therapeutic effect of intracolonically administered nuclear factor κB (p65) antisense oligonucleotide on mouse dextran sulphate sodium (DSS)-induced colitis. *Clin Exp Immunol* 120: 51-58.
58. Inan MS, Tolmacheva V, Wang QS, Rosenberg DW, Giardina C. 2000. Transcription factor NF-κB participates in regulation of epithelial cell turnover in the colon. *Am J Physiol*: G1282-G1291.
59. Erdman SE, Fox GJ, Dangler CA, Feldman D, Horwitz BH. 2001. Cutting edge: Typhlocolitis in NF-κB-deficient mice. *J Immunol* 166: 1443-1447.
60. Iimuro Y, Nishiura T, Hellerbrand C, Behrns KE, Schoonhovlen R, Grisham JW, Brenner, DA. 1998. NF-kappaB prevents apoptosis and liver dysfunction during liver regeneration. *J Clin Invest* 101: 802-811.
61. Thorpe CM, Hurley BP, Lincicome LL, Jacewicz MS, Keusch GT, Acheson DW. 1999. Shiga toxins stimulate secretion of interleukin-8 from intestinal epithelial cells. *Infect Immun* 67: 5985-5993.
62. Zingarelli B, Yang Z, Hake PW, Denenberg A, Wong HR. 2001. Absence of endogenous interleukin 10 enhances early stress response during post-ischaemic injury in mice intestine. *Gut* 48: 610-622.

3

DIFFERENTIAL MODULATION OF ENTEROCYTE-LIKE CACO-2 CELLS AFTER EXPOSURE TO SHORT-CHAIN FATTY ACIDS

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Abstract

The response of intestinal epithelial cells to short-chain fatty acids (SCFAs), which are increasingly used as food additives, was investigated. Human small intestinal epithelial cell model Caco-2 cells were exposed to formate, propionate and butyrate to assess their effect on cellular growth, metabolism, differentiation and protection against bacteria. The Caco-2 cells were entirely grown in the different SCFAs and respective growth patterns were determined. Differentiated cells were exposed to 0-20 mM SCFAs for 48 h and changes in DNA-, RNA-, (glyco)protein syntheses, sucrase isomaltase activity, transepithelial electrical resistance (TEER) and protection against *Salmonella enteritidis* were measured. The SCFAs altered linearly and differentially the growth pattern ranging from stimulation by formate to inhibition by butyrate. Formate inhibited cellular metabolism. Low concentrations of up to 5 mM propionate and 2 mM butyrate stimulated metabolism, while higher doses were inhibitory. Formate had no effect on sucrase isomaltase enzyme activity and TEER, whereas propionate and butyrate increased these markers of differentiation. Infection with *S. enteritidis* did not benefit from the SCFA-induced TEER. It is concluded that formate, propionate and butyrate selectively and differentially modulate growth characteristics, cellular metabolism, sucrase isomaltase activity and TEER in a concentration- and carbon atom-related fashion. The SCFA-induced TEER does not confer protection against *S. enteritidis.*

Introduction

Short-chain fatty acids (SCFAs) are normally present *in vivo* in the colon lumen as major products of anaerobic fermentation of dietary fibers[1]. They are the major source of energy for the colonic mucosa and are readily taken up and preferred by the colonocytes over amino acids, glucose and ketone bodies[2,3]. They, therefore, play an important role in many energy-dependent processes. Their presence in the colon contributes to modulation of biological processes such as cellular metabolism, growth, differentiation and immunity[4,5]. Findings on these processes in SCFA-treated colonocytes are, however, full of discrepancies. On the one hand, SCFAs have been reported to up-regulate metabolism of colonic epithelial cells exemplified by stimulated DNA, RNA and protein syntheses leading to promoted cellular growth and inhibited or delayed differentiation[5,6]. Their deficiency could be associated with colonic atrophy. On the other hand, they induce growth arrest and differentiation with a possible decline in metabolism[4,7]. The latter phenomenon is mainly mediated by butyrate and is generally becoming well understood.

SCFAs' deficiency, especially that of butyrate, has been linked with the pathogenesis of ulcerative colitis, an inflammatory bowel disease. Restoration of the normal endogenous SCFA levels by intracolonic infusion is used in the treatment of ulcerative colitis[8,9].

Although the precise mechanisms are not fully elucidated, SCFAs are known to induce several nuclear changes including inhibition of histone deacetylase, DNA hypermethylation, modification of high mobility groups and down-regulation of epidermal growth factor receptor expression[10]. Of these changes, inhibition of histone deacetylase and the subsequent histone hyperacetylation is the best established mechanism through which SCFAs, especially butyrate, impart colonocytic growth arrest[11]. Through this mechanism, SCFAs are able to turn on several genes responsible for growth and differentiation.

The SCFAs are increasingly used as food additives. To study the interaction of food additives such as SCFAs with small intestinal cells, several models are convenient. Clearly, the systemic effects of a diet supplemented with food additives can only be studied in human or animals[12-14] or in animals with a self-emptying jejunal loop or self-emptying blind pouch[15]. Organ culture of small intestinal tissue has also been used as an *in vitro* model to study the interaction of food additives with tissues[16,17], but this model has a serious drawback. Following its removal from the animal, the intestinal tissues are particularly rapidly degraded, which restricts the use of this model only to studies of short-term food additive effects *in vitro*. In cultures of enterocyte-like Caco-2 cells, it is possible to

investigate both short- and long-term effects of food additives at the level of the individual cells[18-21].

In the present study, we have focused on the SCFAs: formate, propionate and butyrate. These food additives are known to interfere with various biological processes in jejunal and colon cells. We first established the growth characteristics of Caco-2 cells grown in various concentrations of SCFAs. Next, we used 19-day old fully differentiated Caco-2 cells that display characteristics of small intestinal enterocytes both structurally and functionally to investigate if SCFAs induce changes in the cellular metabolism, in differentiation and in transepithelial electrical resistance (TEER) of these *in vitro* counterparts of mature villus enterocytes. In addition, we designed experiments to examine whether Caco-2 cell monolayers showing an improved mucosal integrity (in terms of an increase in TEER) were better protected against invasion by pathogenic bacteria.

Materials and methods

Cell culture

Caco-2 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% (v/v) non-essential amino acids, 10 mM $NaHCO_3$, 1.7 mM glutamine, 0.05 mg ml^{-1} gentamicin, 25 mM HEPES (all Flow Laboratories, Amstelstad B.V., Amsterdam, The Netherlands) and 20% (v/v) fetal calf serum (FCS) (Sanbio B.V., Uden, The Netherlands). Cell cultures were maintained at 37°C in 95% air/5% CO_2 in a humidified atmosphere with three cell culture medium changes per week. Cells were seeded at 40 000 cells cm^{-2} in 24 well plates (2 cm^2/well) or 25 cm^2 tissue culture flasks (both Greiner, Alphen a/d Rijn, The Netherlands) containing 0.5 or 5 ml cell culture medium, respectively. In experiments with filter-grown cells (seeded at 60 000 cells cm^{-2}) or differentiated Caco-2 cells to determine the interaction of SCFAs with the cellular metabolism, the percentage of FCS was gradually reduced and replaced by Ultroser G (Gibco Europe B.V., Hoofddorp, The Netherlands). This reduction was accomplished by replacing cell culture medium containing 20% (v/v) FCS with medium containing 10% (v/v) FCS and 1% (v/v) serum substitute on day 17. One day later (day 18), medium containing 1% (v/v) FCS and 2% (v/v) Ultroser G was substituted by the previous one. This study encompassed 21 passages of the cell line ranging from the 15th to the 35th.

Growth characteristics and cytotoxicity of Caco-2 cells in the presence of SCFAs

Cells seeded at 40 000 cells cm^{-2} in tissue culture 24 flat-bottom well plates (2 cm^2/well) (Gibco) containing 1 ml culture medium were allowed to attach to the plates for 16 h. Subsequently, the medium was replaced by medium containing formate, propionate or butyrate at 0 (control), 1, 2, 5, 10 and 20 mM concentrations. Cell growth and cytotoxicity were monitored while growing in those concentrations for 2, 3, 4, 5, 6, 9, 12, 16 and 19 days. To determine growth at these respective time points, the cells were washed once with 1 ml cell culture medium devoid of FCS and the respective SCFA concentrations. Subsequently, cells were quantified using a rapid tetrazolium-based colorimetric assay[22].

Cell toxicity was measured by means of lactate dehydrogenase (LDH) release from Caco-2 cells[23]. Cells were washed twice with 1 ml 0.01 M PBS, pH 7.3 (each wash 10 min), and 1 ml distilled water was added to the cells. Ultrasonication was done twice at an amplitude of 24 μm for 15 s (30-s interval) with an MSE Soniprep 150 (Beun de Ronde B.V., Abcoude, The Netherlands). Specific intracellular LDH activity expressed as mU mg^{-1} protein was measured in the cell pellet collected from respective wells and expressed as the relative LDH activity (the factor by which the activity was increased or decreased compared with cell cultures that were not incubated with SCFAs).

Incorporation of precursors for DNA, RNA, protein and glycoprotein syntheses by differentiated Caco-2 cells after incubation with SCFAs

Differentiated 19-day-old Caco-2 cells were used to test the effect of 0 (control), 1, 2, 5, 10 and 20 mM SCFA concentrations on the cellular metabolism. Incubation of the cells was performed in 0.5 ml plain DMEM in quadruplicate cultures. After incubation for 44 h, 100 μl plain DMEM containing 0.05 μCi [2-^{14}C]thymidine and 2 μCi D-[6-^{3}H]glucosamine or 0.05 μCi [U-^{14}C]uridine and 2.0 μCi L-[methyl-^{3}H]methionine) was added to the cell monolayer and the incubation was continued further for 4 h (thymidine, glucosamine, uridine and methionine were from Amersham Nederland B.V., Houten, The Netherlands). To stop the incorporation of the radioactive label, 1 ml 10% (w/v) trichloroacetic acid (0^0C) was added to the cells. Subsequently, the cells were washed twice with 1 ml 0.01 M PBS (0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, 0.9% (w/v) NaCl), pH 7.3 (each wash 10 min), fixed with 1 ml methanol for 10 min, and dissolved in 0.5 ml 0.1 N NaOH. The incorporated radioactivity present in 0.2 ml 0.1 N NaOH was determined by liquid scintillation counting in 2 ml Dynagel (J.T. Baker Chemical B.V., Deventer, The Netherlands) as scintillant and performed with a Beckman LS

1701 (Beckman Instruments B.V., Mijdrecht, The Netherlands). The incorporated radioactivity was calculated as disintegration min^{-1} μg^{-1} protein and expressed as the relative incorporation (the factor by which the incorporation was increased or decreased compared with cell cultures that were not incubated with the additives).

Before protein determination[24], cells from separate 24 flat-bottom well plates were washed twice with 1 ml 0.01 M PBS, pH 7.3 (10 min each wash). A total of 1 ml distilled water was added to the cells followed by ultrasonication (twice) at an amplitude of 24 μm for 15 s (30-s interval) with an MSE Soniprep 150.

Sucrase isomaltase activity of differentiated Caco-2 cells after exposure to SCFAs

Fully differentiated (19-day-old) cells grown in 25 cm^2 tissue culture flasks (Costar Europe Ltd, Badhoevedorp, The Netherlands) were exposed to 0 (control), 1, 2 and 10 mM SCFA concentrations for 48 h. Hereafter, the cell monolayers were rinsed twice with 5 ml 0.01 M PBS, pH 7.3, and then incubated during 30 min at 37^0C with 5 ml transfer medium, pH 7.3 (0.8% (w/v) NaCl, 0.02% (w/v) KH_2PO_4, 0.02% (w/v) Na_2EDTA). Dispersed cells were collected in tubes and washed twice with 10 ml 0.01 M PBS, pH 7.3 (0^0C). The washing procedure involved centrifugation (10 min 300*g* at 4°C) and discarding of supernatants. After the last washing step, the cells were collected by centrifugation (10 min 600*g* at 4°C) and used to prepare an enriched fraction of brush border membranes according to a method described by Pinto et al (1983)[25]. Subsequently, the isolated brush border membrane pellets were sonicated twice at 0°C for 15 s, separated by a 30-s interval, at an amplitude of 24 μm with an MSE Soniprep 150. The protein content of the resulting sonicates was determined[24], adjusted to approximately 250 μg protein ml^{-1} and used to measure sucrase isomaltase activity[26] (EC 3.2.1.48). The sucrase isomaltase activity was measured with saccharose as substrate (1 unit = 1 μmol disaccharide hydrolysed min^{-1}) and expressed as enzyme units g^{-1} protein.

Salmonella enteritidis

S. enteritidis 857 was grown on Luria-Bertani (LB) agar and one colony was inoculated into 10 ml LB broth. After growing this inoculum overnight (16 h) with shaking (200 rpm) at 37°C, 100 μl of the resulting bacterial suspension were inoculated into 10 ml LB broth and incubated with shaking (200 rpm) at 37°C for 2 h to obtain logarithmically growing bacteria. A bacterial suspension was made in 10 ml plain DMEM (FCS and gentamicin free) after collection of the bacteria by centrifugation (15 min 1 500*g* at 22°C).

Interference of the TEER of filter-grown differentiated Caco-2 cells by SCFAs and/or S. enteritidis

To determine the effect of SCFAs and/or bacteria on the integrity of the Caco-2 cell, monolayer cells seeded at 60 000 cells cm^{-2} were grown on Transwell polycarbonate filter inserts (growth area 1 cm^2; 0.4 µm pore size; apical volume 600 µl; basolateral volume 1500 µl) (Costar Europe). Since incubation of Caco-2 cells with SCFAs and *S. enteritidis* was performed in plain DMEM (FCS and gentamicin free), the percentage of FCS was gradually reduced and replaced by serum substitute Ultroser G.
The integrity of the cell monolayer was verified by measuring the TEER using a Millicell-ERS Volt/Ohm meter (Millipore Corporation, Bedford, MA, USA). This device contained a pair of chopstick electrodes that facilitated the measurement. The TEER of 19-day-old cells amounted to 225 ± 11 Ohm cm^{-2} after subtracting the resistance of blank filters.

After being exposed for 48 h to 0 (control), 1 and 2 mM SCFAs, the 19-day-old Caco-2 cell monolayers were first equilibrated in plain DMEM (FCS and gentamicin free) for 2 h under cell culture conditions and the TEER was measured for the first time. Subsequently, Caco-2 cells were incubated with *S. enteritidis* 857 (200 bacteria/cell) in plain DMEM (FCS and gentamicin free). The exposure was in such a way that 60 µl apical DMEM were removed and replaced by the same volume of DMEM containing the bacteria. Cells were exposed to bacteria in triplicate for 1 h at 37°C. At the end of exposure the cell monolayers were washed twice with 1 ml gentamicin-containing plain DMEM and allowed to recover for 2 h before determination of TEER.

Statistical analysis

Statistical significance between the mean values of control and SCFA and *S. enteritidis*-exposed cells was assessed by one-way analysis of variance (ANOVA) plus comparison of means. Differences were considered significant at 95% confidence interval using the Student's T-test.

Results

Growth characteristics and cytotoxicity of Caco-2 cells grown in various concentrations of SCFAs

The growth curves of Caco-2 cells grown for 19 days in the absence (dotted line) or presence of various concentrations of formate, propionate or butyrate were different (Fig 3.1). It is obvious that SCFAs altered dose-dependently the growth curve of Caco-2 cells with varying potencies. With increasing numbers of carbon atoms, the SCFAs showed an increasing potency to inhibit cell proliferation. Formate stimulated proliferation of the cells at all doses, although beyond day 12 cell proliferation was inhibited by 20 mM. Low doses of 1 and 2 mM propionate revealed a significant stimulation of cell proliferation, while doses of 10 and 20 mM significantly inhibited proliferation. The lowest dose of butyrate (1 mM) clearly stimulated cell growth. Butyrate concentration of 2 mM had little or no effect at all on Caco-2 cell proliferation and doses of 10 and 20 mM strongly inhibited growth. Stimulation of cell growth was at its height for crypt-like, 6-day-old Caco-2 cells.

Taking the specific activity of intracellular LDH for control cells to be 100, the relative intracellular LDH values for Caco-2 exposed to the various SCFAs did not change significantly (Table 3.1). The LDH released from the cells therefore did not vary between the control and treated cells.

Changes in the cellular metabolism of differentiated Caco-2 cells after exposure to SCFAs

Considerable changes in the relative incorporation of ^{14}C-thymidine, ^{14}C-uridine, ^{3}H-methyl methionine and ^{3}H-glucosamine were observed when differentiated Caco-2 cells were incubated for 48 h in the presence of formate, propionate or butyrate (Fig 3.2). All concentrations of formate used here significantly inhibited the cellular metabolism in a dose-related manner.

Fig 3.2 also shows the effect of propionate on differentiated Caco-2 cellular metabolism. Low concentrations of propionate (1 and 2 mM) significantly stimulated the metabolic parameters. The 5-mM dose only revealed stimulation of RNA and glycoprotein syntheses, whereas concentrations of 10 and 20 mM inhibited the cellular metabolism.

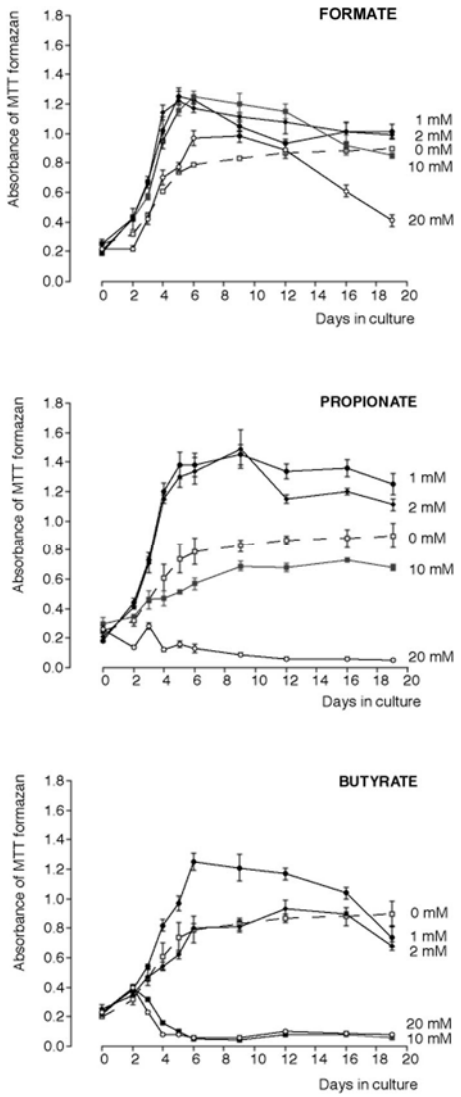

Fig 3.1. Caco-2 cell growth curve for cells grown in the presence of SCFAs. *Three different cell passages have been used to establish the effect of SCFAs. For each cell passage, the Caco-2 cell growth curve was determined using quadruplicate cultures. The dotted line represents the growth curve of Caco-2 cells not exposed to SCFAs.*

Table 3.1 Relative intracellular lactase dehydrogenase activity of enterocyte-like Caco-2 cells exposed to SCFAs.

Days in culture	0 mM	1 mM	20 mM
Formate			
2	100.0 ± 3.0	99.5 ± 2.3	98.9 ± 3.6
5	100.0 ± 2.6	97.3 ± 2.2	99.0 ± 1.7
19	100.0 ± 2.4	97.5 ± 2.4	96.6 ± 2.8
Propionate			
2	100.0 ± 2.4	99.8 ± 1.4	97.7 ± 3.4
5	100.0 ± 2.8	97.9 ± 2.7	97.4 ± 3.5
19	100.0 ± 1.3	99.1 ± 2.6	96.9 ± 4.0
Butyrate			
2	100.0 ± 3.3	99.6 ± 1.8	97.2 ± 4.0
5	100.0 ± 4.0	98.7 ± 3.3	98.5 ± 2.2
19	100.0 ± 2.4	101.3 ± 3.8	96.7 ± 1.9

Three cell passages and triplicate cultures per passage have been used to establish the effect of SCFAs on the specific intracellular lactate dehydrogenase activity. Results are the mean relative intracellular lactate dehydrogenase activity ± SD. The results did not reveal any significant differences.

The metabolism of differentiated Caco-2 cells exposed to low concentrations of butyrate was also stimulated significantly and was even higher than with the same propionate concentrations. The higher butyrate concentrations (5, 10 and 20 mM) inhibited DNA, RNA, protein and glycoprotein syntheses in a dose-related manner.

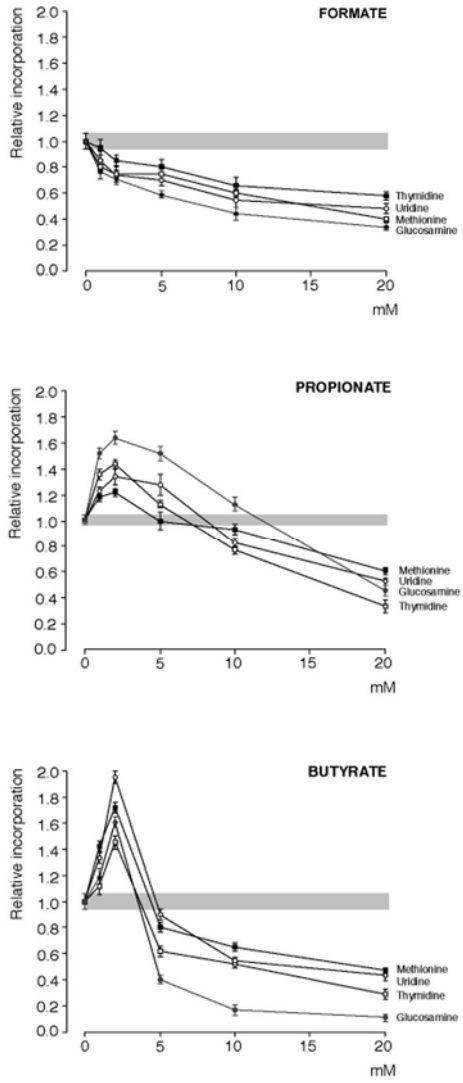

Fig 3.2. Changes in the cellular metabolism of differentiated Caco-2 cells after exposure to SCFAs. *The relative incorporation of thymidine, uridine, methionine and glucosamine has been established after 48-h exposure to SCFAs using three cell passages and quadruplicate cultures per passage. Results are the mean relative incorporation ± SD. The dotted area represents the mean relative incorporation ± SD of cells not exposed to SCFAs.*

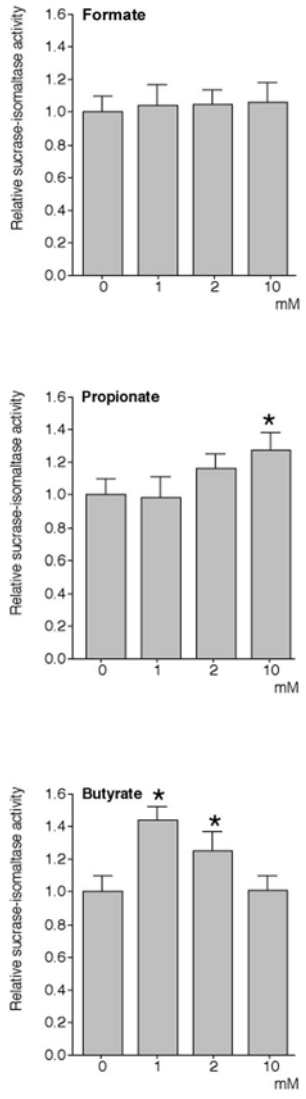

Fig 3.3. Relative sucrase isomaltase activity of differentiated Caco-2 cells exposed to SCFAs. *A brush border membrane-enriched fraction has been used in determining the specific activity of sucrase isomaltase. Three cell passages and triplicate cultures per passage were used to establish the effect of SCFAs. The results are the mean relative sucrase isomaltase activity ± SD. *Significant difference from the cells not exposed to SCFAs ($p < 0.05$).*

Interaction of SCFAs with sucrase isomaltase activity in the brush border membrane of differentiated Caco-2 cells

To establish whether or not SCFAs interfere with the differentiation of Caco-2 cells, the enzyme activity of the brush border enzyme sucrase isomaltase was measured in control Caco-2 cells and in cells exposed for 48 h to 0 (control), 1, 2 and 10 mM concentrations (Fig 3.3). Compared with differentiated Caco-2 cells not exposed to formate, there was little or no change in sucrase isomaltase activity after exposure. The changes in the enzyme activity of all tested concentrations were marginal and not significant. In contrast to these effects, exposure to propionate and butyrate caused a significant increase in sucrase isomaltase activity. In comparison with control Caco-2 cells, cells exposed to 10 mM propionate showed a marked increase in enzyme activity. With respect to butyrate, the sucrase isomaltase activity was significantly highest at 1 mM butyrate, lower but still significant at 2 mM, and became non-significant at 10 mM. It appeared that the longer the carbon chains of SCFAs, the higher the increase in enzyme activity.

Interference of SCFAs with the TEER of differentiated Caco-2 cells and protection against bacteria

In order to test whether SCFAs interfere with the mucosal integrity of filter-grown differentiated Caco-2 cells, the TEER was measured in their presence and absence (Fig 3.4). For the tested doses of 1 and 2 mM SCFA, formate did not alter TEER significantly. On the contrary, the very same concentrations of propionate and butyrate significantly increased the TEER in a dose-dependent manner.

To find out that in comparison with control Caco-2 cells, propionate or butyrate exposed cells are better protected against deleterious effects of bacteria, the resistance was measured once again after exposure to *S. enteritidis* 857 (200 bacteria/cell) (Fig 3.4). Exposure of cells to bacteria in the presence of formate revealed a significant decrease in TEER. A similar reduction was found in control Caco-2 cells in the absence of SCFAs. Although propionate and butyrate induced an increase in TEER, this initially higher resistance did not confer protection against *S. enteritidis* 857. After bacterial exposure, the values of TEER were not significantly different from infected control cells.

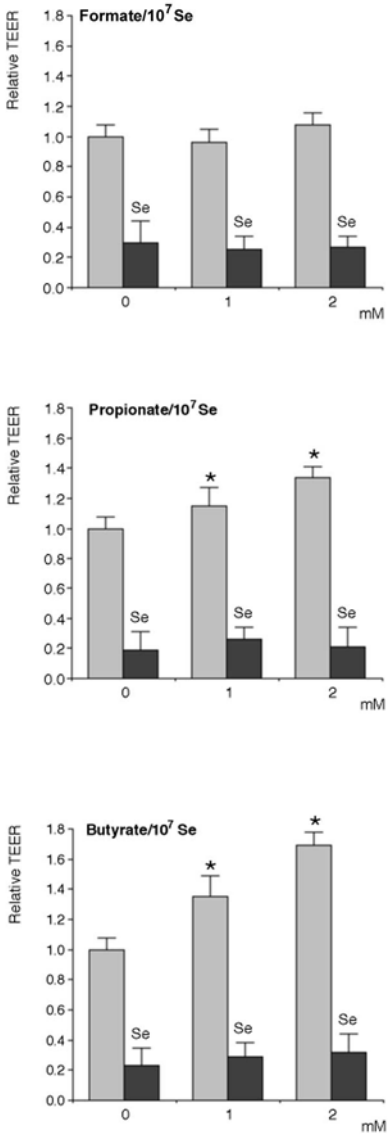

Fig 3.4. Interference of SCFAs with the TEER of filter-grown differentiated Caco-2 cells and infection by Salmonella enteritidis. *Changes in the relative TEER were established using three cell passages and triplicate filter-grown cultures per passage. *Significant increase in the relative TEER compared with cells not exposed to SCFAs ($p < 0.05$), whereas Se represents the relative TEER after apical infection for 1 h with Salmonella enteritidis (10^7 bacteria ml^{-1}).*

Discussion

SCFAs are produced in the colon following bacterial fermentation of carbohydrate diet, particularly fibers. Acetate, propionate and butyrate are the major SCFAs in the colon accounting for 83% of all colonic SCFAs at approximate molar ratio of 60 : 25 : 15[1,27]. Their physiological concentrations vary depending on the amount of dietary fibers. In the normal human colon, physiological butyrate concentrations range from 5 mM to 24 mM[28,29]. Our results in table 1 clearly show no difference between control and cells exposed to as high as 20 mM SCFAs in terms of LDH release. This alludes that these concentrations may not be toxic to Caco-2 cells under these conditions. Since *in vivo* butyrate concentrations of up to 150 mM are non-toxic to rat intestinal cells[6] and so are 20 and 10 mM to cultured human intestinal cells HT-29[10] and Caco-2 cells[30], respectively, it is very unlikely indeed that the concentrations used in this study (1-20 mM) are toxic to enterocyte-like Caco-2 cells.

When enterocyte-like Caco-2 cells were grown in the presence of SCFAs for 19 days, it was observed that differences in chemical structure (number of carbon atoms) and concentration accounted for variations of the growth pattern (Fig 3.1). All formate concentrations stimulated cell proliferation in the logarithmic phase as well as in the beginning of the stationary phase of cell growth. Butyrate appeared to be the most potent inhibitor of cell proliferation. With the exception of the lowest dose of butyrate (1 mM), which stimulated cell proliferation in the logarithmic as well as the stationary phase, the other concentrations had either little or no effect at all on cell proliferation (2 mM) or were strongly inhibitory (10 and 20 mM). Propionate shows growth characteristics that are intermediate between formate and butyrate. Again the stimulatory effect of propionate (1 and 2 mM) is most obvious in the logarithmic and stationary phase of cell growth, whereas high doses (10 and 20 mM) clearly displayed an inhibitory response. The SCFA-induced growth stimulation of normal colonic epithelial cells observed by others is accompanied by an increase in mitotic index, which is an indication of induced cell proliferation[31]. While the effects of formate are not established, propionate and butyrate concentrations of up to 40 mM stimulate growth in the normal rat colon[6]. By contrast, cell growth arrest is the potent SCFA-induced growth effect in transformed colonocytes[4,7,21]. The latter paradoxical effect is observed in most colon cancer cells and has granted SCFAs especially butyrate an antitumorigenesis potentiality. Consistently, our study suggests that lower SCFAs (such as formate) are growth stimulants while higher SCFAs (propionate and butyrate) are inhibitors at relatively higher concentrations.

The changes in DNA, RNA, protein and glycoprotein syntheses induced in 19-day-old, villus-like differentiated Caco-2 cells after incubation with SCFAs

were considerable (Fig 3.2). The incorporation studies clearly showed that exposure to all formate concentrations significantly inhibited the cellular metabolism of the cells. Low concentrations of propionate and butyrate led to stimulation of the cellular metabolism, whereas high concentrations of these SCFAs displayed an inhibitory effect. It is interesting and undoubtedly striking that the effect exerted on the cellular metabolism appears to be concentration dependent and that butyrate had higher stimulatory potency over propionate.
Observations such as these in which case SCFAs stimulated or inhibited DNA and protein syntheses have also been reported for colonocytes[32,33]. Because formate, propionate and butyrate have one, three and four carbon atoms, respectively, this difference in the SCFA chemical structure may also account for the variations in the stimulation of cellular metabolism. These structural differences are known to influence their absorption, readiness of hydrogen ion dissociation and the subsequent intracellular pH[34].

To our knowledge, the effect of formate on enterocyte-like Caco-2 cells shown here is a first report and thus comparable results are sketchy. It has been established that formate is a free radical scavenger that plays an important role in prevention, protection and restitution of cell membrane functions[35]. This SCFA is also involved in NAD reduction and serves as a respiration substrate by acting on cytochrome oxidase[36,37]. The involvement of formate in both DNA and RNA metabolism[38] could be important in the proliferation of Caco-2 cells observed in this study. Unlike the well-established mechanism of propionate and butyrate on histone hyperacetylation, formate, a SCFA with neither an acetyl nor a methyl group, may thus induce genetic transcription activation in a different pathway possibly predominated by growth promotion. The other SCFAs are known to induce histone hyperacetylation and DNA methylation[10,11] to turn on various genes involved in cell cycle that determine cellular proliferation and differentiation. Such genes include oncogenes (*Ha-ras, c-src* and *lck*), cyclin D1, c-myc, p21 and urokinase-type plasminogen activator genes[39-41]. By modulating the expression of these genes, SCFAs interfere with progression of the cell cycle in different ways. Butyrate, for instance, represses expression of the cyclin D1 gene to inhibit the exit from G0/G1 in the cell cycle. The exit is normally governed by D-type cyclins such as D1, D2 and D3 induced at the beginning of the G1 phase[42,43]. Inhibition of this exit leads to growth arrest and promotion of differentiation[41]. Butyrate also decreases expression of urokinase-type plasminogen activator and c-myc genes and induces expression of p21 to inhibit cellular proliferation[44]. In reality, induction of p21 cell cycle inhibitor is thought to be critically important in butyrate-mediated growth inhibition of colon cancer cells[44].

Concurrent with growth arrest or stimulation is promotion of or delayed differentiation, respectively. In this study, we did not observe eminent

differentiation discrepancies. Instead, our data showed a clear growth and differentiation relationship (Fig 3.1, 3.3 and 3.4). We observed that formate, which promoted growth (Fig 3.1), induced neither sucrase isomaltase activity (Fig 3.3) nor TEER (Fig 3.4), implying that it inhibited or delayed differentiation. Butyrate-induced effects were the antithesis of those of formate, while propionate showed intermediate upshots. The induction of differentiation by SCFAs in particular butyrate is common in colonocytes[4,7,11]. It is mediated via histone hyperacetylation or DNA methylation that eventually modulates expression of genes coding for differentiation. One such inflect is the reduced expression of AT motif binding factor 1-A (ATBF1-A). The ATBF1-A negatively regulates transcription of the brush border enzyme gene, aminopeptidase-N, and is probably a landmark of enterocyte differentiation[45]. Its transcriptional repression may pave way for transcriptional activation of brush border membranes such as sucrase isomaltase. The observed increase in the activity of this enzyme in this study could be a result of this phenomenon. Another possible mechanism by which SCFAs may induce differentiation is through triggering cAMP activity. Triggered cAMP activity results in elevated intracellular cAMP levels that are known to increase TEER[46]. Existing evidences on butyrate-induced increase in intracellular cAMP[47] could account for a possible mechanism of SCFA-induced TEER. The increased TEER or reduced paracellular permeability could be a result of increased tight junction functions. This may involve activation of genes coding for tight junction specific proteins like cingulin, occludin, ZO-1 and ZO-2[48].

Promotion of differentiation phenotype in particular the increase in TEER (Fig 3.4) could have protective significance against development of pathological processes in the colonic mucosa. The intestinal epithelium functions as a first line of defence because of its barrier integrity that separates the sterile host environment from the pro-inflammatory macromolecules in the intestinal lumen. Induction of TEER can partly add to the epithelial barrier integrity and thereby contribute to intestinal protection against pathogens such as bacteria. A link between enteritis and TEER,[8] the efficiency of butyrate enemas in preventing Crohn's disease/colitis[9] and the failure of the enteroinvasive *Yersinia pseudotuberculosis* to invade differentiated as opposes to undifferentiated Caco-2 cells[49] are some of the SCFA-mediated protective processes. By testing the hypothesis whether SCFA-induced TEER prevents bacterial invasion, we found that the SCFA-induced TEER does not confer protection against *S. enteritidis* invasion (Fig 3.4). This was demonstrated by a decrease in TEER following exposure to the bacterium. Explanation for this awaits further studies. However, SCFAs are known to inhibit intestinal trefoil factor gene coding for trefoil peptides[50]. Trefoil peptides are a small family of protective polypeptides that stimulate cell migration to institute cellular repair following injury. Down-regulation of trefoil members proceeds to

inhibition of cell migration and a possible barrier distortion[50]. It is possible that the observed failure of decreased paracellular permeability to confer protection against *S. enteritidis* was partly an effect of SCFAs on cell migration that maintains barrier integrity. In addition, the establishment of tight junctions takes a considerably longer time. Although TEER did not confer protection, it should be born in mind that ample time is needed for the establishment of barrier integrity especially the role of tight junctions. In all, the direct protective role of SCFAs to the intestine with regard to promotion of differentiation remains enigmatic.

In summary, we have shown differential and selective modulation of formate, propionate and butyrate on enterocyte-like Caco-2 cell metabolism. Our findings also suggest that formate is a potent growth promotor and inhibitor of differentiation, an effect opposite to that of butyrate. Propionate induces intermediate responses to those mediated by formate and butyrate. The pattern is linear with the increasing number of carbon atoms and is dose dependent. The increase of TEER induced by SCFAs does not confer protection against *S. enteritidis.*

References

1. Cummings JH, Branch WJ. 1990. Fermentation and production of short chain fatty acids in human large intestine. In: *Dietary Fiber: Basic and Clinical Aspects*, ed Vahouny GB, Kritchevesky D. Plenum, NY, 131-152.
2. Roediger WEW. 1982. Utilization of nutrients by isolated epithelial cells of the rat colon. *Gastroenterology* 83: 424-429.
3. Ardawi MSM, Newsholme EA. 1985. Fuel utilization in colonocytes of the rat. *Biochem J* 231: 713-719.
4. Bernard JA, Warwick G. 1993. Butyrate rapidly induces growth inhibition and differentiation in HT-29 cells. *Cell Growth Differ* 4: 495-501.
5. Frankel WL, Zhang W, Singh A, Klurfeld DM, Don S, Sakata T, Modlin I, Rombeau JL. 1994. Mediation of the trophic effects of short-chain fatty acids on the rat jejunum and colon. *Gastroenterology* 106: 375-380.
6. Kripke SA, Fox AD, Berman JM, Settle RG, Rombeau JL. 1989. Stimulation of intestinal mucosal growth with intracolonic infusion of short-chain fatty acids. *J Parent Enter Nutr* 13: 109-116.
7. Medina V, Afonso JT, Alverez-Arguelles H, Hernandez C, Gonzalez F. 1998. Sodium butyrate inhibits carcinoma development in a 1,2-dimethylhydrazine-induced rat colon cancer. *J Parent Enter Nutr* 22: 14-17.
8. Roediger WEW. 1980. The colonic epithelium in ulcerative colitis: an energy deficiency disease? *Lancet* 2: 712-715.
9. Scheppach W, Sommer H, Kirchner T, Paganelli GM, Bartram P, Christl S, Richter F, Dusel G, Kasper H. 1992. Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. *Gastroenterology* 103: 51-56.

10. Archer S, Meng S, Wu J, Johnson J, Tang R, Hodin R. 1998. Butyrate inhibits colon carcinoma cell growth through two distinct pathways. *Surgery* 124: 248-253.
11. Lee DY, Hayes JJ, Pruss D, Wolffe AP. 1993. A positive role for histone acetylation in transcription factor access to nucleosomal DNA. *Cell* 72: 73-84.
12. Kik MJL, Huisman AFB, van der Poel AFB, Mouwen JMVM. 1990. Pathologic changes of the small intestinal mucosa of pigs after feeding *Phaseolus vulgaris* beans. *Vet Pathol* 27: 329-334.
13. Pusztai A, Koninkx J, Hendriks H, Kok W, Hulscher S, van Damme EJM, Peumans WJ, Grant G, Bardocz S. 1996. Effect of the insecticidal Galanthus nivalis agglutinin on metabolism and the activities of brush border enzymes in the rat small intestine. *J Nutr Biochem* 7: 677-682.
14. Wachtershauser A, Stein J. 2000. Rationale for the lumenal provision of butyrate in intestinal diseases. *Eur J Nutr* 39: 164-171.
15. Kik MJL, van den Muysenberg A, van Kleef D. 1995. Effects of *Phaseolus vulgaris* isolectins on the structure and function of the small intestinal mucosa in the self-emptying blind-pouch model in piglets. *J Animal Physiol Anim Nutr* 73: 149-158.
16. Trier JS. 1980. Organ culture of the mucosa of human small intestine. In: *Methods in Cell Biology*, ed Curtis HC, Trump BF, Stoner GD. Academic Press, NY, 365-383.
17. Kik MJL, Koninkx JFJG, van den Muysenberg A, Hendriksen F. 1991. Pathological effects of *Phaseolus vulgaris* isolectins on pig jejunal mucosa in organ culture. *Gut* 32: 886-892.
18. Koninkx JFJG, Hendriks HGCJM, van Rossum JMA, van den Ingh TSGAM, Mouwen JMVM. 1992. Interaction of legume lectins with the cellular metabolism of differentiated Caco-2 cells. *Gastroenterology* 102: 1516-1523.
19. Koninkx JFJG, Brown DS, Kok W, Hendriks HGCJM, Pusztai A, Bardocz S. 1996. Polyamine metabolism of enterocyte-like Caco-2 cells after exposure to *Phaseolus vulgaris* lectin. *Gut* 38: 47-52.
20. Mariadason JM, Barkla DH, Gibson PR. 1997. Effect of short-chain fatty acids on paracellular permeability in Caco-2 intestinal epithelium model. *Am J Physiol* 272: G705-G712.
21. Basson MD, Emenaker NJ, Hong F. 1998. Differential modulation of human (Caco-2) colon cancer cell line phenotype by short chain fatty acids. *Proc Soc Exp Biol Med* 217: 476-483.
22. Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH, Boyd MR. 1988. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res* 48: 589-601.
23. Unno N, Fink MP. 1998. Intestinal epithelial hyperpermeability: Mechanisms and relevance of disease. *Gastroenterol Clin North Am* 27: 289-307.
24. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic acid. *Anal Biochem* 150: 76-85.
25. Pinto M, Robine-Leon S, Appay MD, Kedinger M, Triadou N, Dussaulx E, Lacroix B, Simon-Assmann P, Haffan K, Fogh J, Zweibaum A. 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma Caco-2 cell line in culture. *Biol Cell* 47: 323-330.
26. Messer M, Dahlqvist A. 1966. A one step ultramicromethod for the assay of intestinal disaccharidases. *Anal Biochem* 14: 376-392.
27. Nyman M, Aso NG. 1982. Fermentation of dietary fiber components in rat intestinal tract. *Br J Nutr* 47: 357-366.
28. Cummings JH, Pomare EW, Branch WJ, Naylor CPE, MacFarlane GT. 1987. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* 28: 1221-1227.
29. Treem WR, Ahsan N, Shoup M, Hyams J. 1994. Fecal short-chain fatty acids in children with inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 18: 159-164.

30. Mariadason JM, Velcich A, Wilson AJ, Augenlicht LH, Gibson PR. 2001. Resistance to butyrate-induced cell differentiation and apoptosis during spontaneous Caco-2 cell differentiation. *Gastroenterology* 120: 889-899.
31. Sakata T, Yajima T. 1984. Influence of short chain fatty acids on the epithelial cell division of the gastrointestinal tract. *Q J Exp Physiol* 69: 639-648.
32. Marsman KE, McBurney MI. 1996. Dietary fiber and short-chain fatty acids affect cell proliferation and protein synthesis in isolated rat colonocytes. *J Nutr* 126: 1429-1437.
33. Emenaker NJ, Basson MD. 2001. Short chain fatty acids differentially modulate cellular phenotype and c-myc protein levels in primary human nonmalignant and malignant colonocytes. *Dig Dis Sci* 46: 96-105.
34. DeSoignie R, Sellin JH. 1994. Propionate-initiated changes in intracellular pH in rabbits colonocytes. *Gastroenterology* 107: 347-356.
35. Wustmann C, Fischer HD, Schmidt J. 1986. Protective and restitutive effects of antihypoxic drugs on posthypoxic dopamine release inhibition. *Biomed Biochem Acta* 45: 549-556.
36. Zakharova EV. 1979. Effect of formate on the respiration of different microorganisms. *Mikrobiologiia* 48: 604-609.
37. Pinchukova EE, Varfolomeev SD. 1980. Reversible oxidation-reduction of NAD by hydrogen, catalysed by soluble hydrogenase from *Alcaligenes eutrophus* Z-1. *Biokhimiia* 45: 1405-1411.
38. Casti A, Morini G, Reali N, Fior TD, Rabbi A. 1976. Effect of carbenoxolone (CBO) on metabolism of nucleic acids, polyamine and proteins of gastric mucosa '*in vivo*' and '*in vitro*'. *Ateneo Parmense Acta Biomed* 47: 47-58.
39. Heruth DP, Zirnstein GW, Bradley JF. 1993. Sodium butyrate causes an increase in the block to transcriptional elongation in the c-myc gene in SW837 rectal carcinoma cells. *J Biol Chem* 268: 20466-20472.
40. Steinman RA, Hoffman B, Iro A, Guillouf C, Liebermann DA, El-Houseini ME. 1994. Induction of p21 (WAF-1/CIP1) during differentiation. *Oncogene* 9: 3389-3396.
41. Lallemand F, Courilleau D, Sabbah M, Redeuilh G, Mester J. 1996. Direct inhibition of the expression of cyclin D1 gene by sodium butyrate. *Biochem Biophys Res Commun* 229: 163-169.
42. Yoshida M, Beppu T. 1988. Reversible arrest of proliferation of rat 3Y1 fibroblasts in both the G1 and G2 phases by trichostatin A. *Exp Cell Res* 177: 122-131.
43. Baldin V, Lukas J, Marcotte MJ, Pagano M, Draetta G. 1993. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev* 7: 812-821.
44. Archier SY, Meng S, Shei A, Hodin RA. 1998. $p21^{WAF1}$ is required for butyrate-mediated growth inhibition of human colon cancer cells. *Proc Natl Acad Sci USA* 95: 6791-6796.
45. Kataoka H, Joh T, Miura Y, Tamaoki T, Senoo K, Ohara H, Nomura T, Tada T, Asai K, Kato T, Itoh M. 2000. AT motif binding 1-A (ATBF1-A) negatively regulates transcription of the aminopeptidase N gene in the crypt-villus axis of small intestine. *Biochem Biophys Res Commun* 267: 91-95.
46. Duffey ME, Hainau B, Ho S, Bentzel CJ. 1981. Regulation of epithelial tight junction permeability by cyclic AMP. *Nature* 294: 451-453.
47. Prasad KN. 1980. Butyric acid: a small fatty acid with diverse biological functions. *Life Sci* 27: 1351-1358.
48. Anderson JM, van Itallie CM. 1995. Tight junctions and the molecular basis for regulation of paracellular permeability. *Am J Physiol* 269: G467-G475.
49. Coconnier MH, Bernet-Camard MF, Servin AL. 1994. How intestinal epithelial cell differentiation inhibits the cell-entry of Yersinia pseudotuberculosis in colon carcinoma Caco-2 cell line in culture. *Differentiation* 58: 87-94.
50. Tran CP, Familari M, Parker LM, Whitehead RH, Giraud AS. 1998. Short-chain fatty acids inhibit trefoil factor gene expression in colon cancer cells. *Am J Physiol* 275: G85-G94.

4

ANTI-INFLAMMATORY PROPERTIES OF HSP70 AND BUTYRATE ON SALMONELLA-INDUCED IL-8 SECRETION IN ENTEROCYTE-LIKE CACO-2 CELLS

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Abstract

Intestinal epithelial cells secrete the chemokine interleukin (IL)-8 in the course of inflammation. Because heat shock proteins (Hsps) and butyrate confer protection to enterocytes we investigated whether they modulate *Salmonella enteritidis*-induced secretion of IL-8 in enterocyte-like Caco-2 cells. Undifferentiated crypt-like and differentiated villus-like Caco-2 cells incubated with or without butyrate (0-20 mM, 48 h) were infected with *S. enteritidis* after (1 h at 42°C, 6 h at 37°C) or without prior heat shock (1 h at 37°C, 6 h at 37°C). Levels of Hsp70 production and IL-8 secretion were analyzed using immunostaining of Western blots and ELISA, respectively. The Caco-2 cells secreted IL-8 in response to *S. enteritidis* and produced Hsp70 after heat shock or incubation with butyrate. The IL-8 secretion was inhibited by heat shock and butyrate concentrations as low as 0.2 mM for crypt-like and 1 mM for villus-like cells. In a dose-dependent manner, higher butyrate concentrations enhanced IL-8 secretion to maximal levels followed by a gradual but stable decline. This decline was associated with increasing production of Hsp70 and was more vivid in crypt-like cells. In addition, the higher concentrations abolished the heat shock inhibitory effect. Instead, they promoted the IL-8 production in heat shocked cells even in the absence of *S. enteritidis*. We conclude that heat shock and low concentrations of butyrate inhibit IL-8 production by Caco-2 cells exposed to *S. enteritidis*. Higher butyrate concentrations stimulate the chemokine production and override the inhibitory effect of the heat shock. The IL-8 down-regulation could in part be mediated via production of Hsp70.

Introduction

The involvement of the intestinal epithelium in the host defense is well apprehended. It serves as an effective physical barrier to protect the internal host milieu against various potentially harmful luminal contents. The enterocytes of this epithelium secrete an array of proinflammatory cytokines (interleukin (IL)-1β, IL-6, IL-8, monocyte chemoattractant protein-1 and tumor necrosis factor-α) either constitutively[1,2] or upon stimulation by inflammatory agents including proinflammatory cytokines (IL-1β and tumor necrosis factor-α) from other sources and pathogenic bacteria[3]. These cytokines in turn induce a local defensive inflammatory response. The chemoattractant cytokine IL-8 is pivotal to and governs the progress of most local intestinal inflammations. IL-8 attracts and activates neutrophils at the site of infection. Its secretion leads to neutrophil infiltration, which may subsequently culminate into epithelial cell damage[4]. Therefore, its down-regulation is vitally important in the prevention of chronic inflammation.

Within the intestinal compartment, regulation of IL-8 production can be influenced by luminal components such as short chain fatty acids, which are normally present *in vivo* in the colon lumen as major products of anaerobic fermentation of dietary fibers[5]. Butyrate is the most potent and abundant short chain fatty acid. Its intestinal physiological concentration ranges from 5-24 mM depending upon the dietary fiber composition[6,7]. Butyrate plays an important role in the homeostasis of the intestinal epithelium. It contributes to modulation of biological processes such as cellular metabolism, growth, differentiation and immunity[8,9]. Butyrate deficiency is linked with the pathogenesis of inflammatory bowel diseases[10]. Restoration of the normal endogenous butyrate levels by intracolonic infusion is used in the treatment of these diseases[10,11] but with limited success[12,13]. This limitation may possibly be due to the presence of IL-8 in the intestinal mucosa, especially because butyrate can inhibit[14-16] or stimulate[2,17] IL-8 production by intestinal cells.

Heat shock proteins (Hsps) are a group of evolutionary conserved proteins ranging in size from 8 kDa to 150 kDa that are synthesized rapidly by most cells responding to stress-related events[18,19]. They act as chaperones through protein-protein interaction to protect the already synthesized proteins against further injury. In turn, they protect cells against noxious processes including heat stress, infection and inflammation. Their protection against inflammation is through suppression of proinflammatory cytokine production[20]. The induction of Hsp70 by intestinal cells is known to protect against several stresses including hyperthermia and ischaemia/reperfusion injury[21]. In respiratory epithelial cells, Hsp70 imparts an

anti-inflammatory role by inhibiting IL-8 production[22]. This role has not been established in enterocytes.

Considering the protective nature of Hsps, the range of homeostatic processes influenced by butyrate and the concurrent exposure of intestinal cells to butyrate, it is of interest to know how butyrate and Hsps interact. Because butyrate induces Hsp25 in rat colon cells and in IEC-18 cells (rat intestinal cell line) [23], it is tempting to suggest that butyrate may also induce Hsp70 in human intestinal cells. Subsequently these changes in Hsp70 levels may have effect on IL-8 synthesis. It is therefore reasonable to hypothesize that butyrate modulates the IL-8 secretion by intestinal cells and that this modulation may be mediated, at least in part, via synthesis of Hsps.

In the experiments presented in this study we focused on the anti-inflammatory properties of the heat shock response and butyrate. In particular, we investigated the anti-inflammatory potency of Hsp70, induced by either heat shock or butyrate, on *Salmonella*-induced IL-8 secretion by intestinal cells and established its extent. We used Caco-2 cells, an *in vitro* model of the human intestinal epithelium, which differentiate in culture and acquire characteristics both structurally and functionally of either crypt cells (5-day-old Caco-2 cells) or villus cells (19-day-old Caco-2 cells) of the small intestine[24-26].

Materials and methods

Cell culture

Human colon adenocarcinoma Caco-2 cells (ATCC HTB 37) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% (v/v) non-essential amino acids, 10 mM $NaHCO_3$, 1.7 mM glutamine, 50 µg ml^{-1} gentamicin, 25 mM HEPES (all Flow Laboratories, Amstelstad B.V., Amsterdam, The Netherlands) and 20% (v/v) fetal calf serum (Sanbio B.V., Uden, The Netherlands). Supplemented culture medium devoid of gentamicin and fetal calf serum in the experiments is referred to as plain DMEM. Cell cultures were maintained at 37°C in 95% air/5% CO_2 in a humidified atmosphere with three cell culture medium changes per week. Cells were seeded at 40 000 cells cm^{-2} in 25 cm^2 tissue culture flasks (Greiner, Alphen a/d Rijn, The Netherlands) containing 5 ml cell culture medium.

Heat shock response

To induce the heat shock response, Caco-2 cells grown in 25 cm^2 tissue culture flasks were exposed to 42°C for 1 h. This was achieved by fully immersion of the flasks in a water bath heated by a circulating thermostat DC10 (Haake, Karlsruhe, Germany) which provided a temperature stable within 0.02^0C. Temperature equilibration of the cell monolayers took about 30 s. Cells were then allowed to recover at 37°C in a 95% air/5% CO_2 humidified incubator for 6 h. The cell culture medium and the Caco-2 cells were then collected.

Incubation of Caco-2 cells with butyrate

Caco-2 cells were cultured to crypt-like (day 5) or villus-like (day 19) enterocytes after which they were incubated in culture medium containing 0, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 mM butyrate for 48 h. After the first 24 h, cells were exposed to *Salmonella enteritidis* (1 h) after or without prior induction of the heat shock response. Exposure to butyrate was continued with those cells receiving a heat shock, whereas the subsequent exposure to bacteria took place in the absence of butyrate. The cell culture medium and the Caco-2 cells were then collected.

Salmonella enteritidis

Salmonella enteritidis 857 was grown on Luria-Bertani (LB) agar and one colony was inoculated into 5 ml LB broth. After growing this inoculum overnight (16 h) with shaking (200 rpm) at 37°C, 1 ml of the resulting bacterial suspension was inoculated into 100 ml LB broth and incubated with shaking (200 rpm) at 37°C for 2 h to obtain logarithmically growing bacteria. A bacterial suspension was made in 100 ml plain DMEM after collection of the bacteria by centrifugation (15 min 1500*g* at 22°C). From the bacterial suspension a serial dilution was made and cells were exposed to 0-1000 bacteria/cell for 1 h. To stop the exposure, cells were washed twice with 5 ml plain DMEM supplemented with 50 µg ml^{-1} gentamicin.

Collection of the cell culture medium and the Caco-2 cells

The cell culture medium was collected in tubes and centrifuged (10 min 600*g* at 4°C) to obtain the supernatants. After collecting the cell culture medium, the cell monolayers were rinsed twice with 5 ml 0.01 M phosphate-buffered saline (PBS) (0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, 0.9% (w/v) NaCl), pH 7.3 at 37°C. The monolayers were then incubated for 30 min at 37°C with 5 ml transfer

medium, pH 7.3 (8 g l^{-1} NaCl, 0.2 g l^{-1} KH_2PO_4, 0.2 g l^{-1} $Na_2EDTA.2H_2O$). The dispersed cells were collected in tubes and washed twice with 10 ml 0.01 M PBS, pH 7.3 (0°C). The washing procedure involved centrifugation (10 min 300*g* at 4°C) and discarding of supernatants. After the last washing step the cells were collected by centrifugation (10 min 600*g* at 4°C). The collected cell culture supernatants and the cells were then stored at -70°C until being analyzed.

Western blot analysis for Hsp70

To the collected Caco-2 cells, 0.5 ml (for crypt-like) or 1.0 ml (for villus-like) distilled water (4^0C) was added and the mixture was sonicated at 0^0C for 30 s at an amplitude of 24 µm with an MSE Soniprep 150 (Beun de Ronde BV, Abcoude, The Netherlands). The protein content of the resulting sonicates was determined[27] and found to be equal in all samples. An equal volume of loading buffer (twice the strength) (125 mM tris (hydroxymethyl) aminomethane-HCl, 4% sodium dodecyl sulfate (SDS), 10% ß-mercaptoethanol, 20% glycerol and 0.0015% bromophenolblue, pH 6.8) was added to the protein samples and the mixture was heated at 95^0C for 5 min. After loading the slots of the gel with equal amounts of proteins (10 µg) they were separated by SDS polyacrylamide gelelectrophoresis on 10% gels. A protein ladder (Bio-Rad Laboratories, California, USA) was loaded as a molecular weight marker. Subsequently, the proteins were transferred to Immobilon-P PVDF membrane following the recommendations of the manufacturer (Millipore, Bedford, USA). Protein transfer was confirmed by staining with Ponceau Red stain. The non-specific binding sites on the membrane were blocked by incubating the membrane for 1 h with 10 ml blocking solution (0.1% Tween/PBS, pH 7.3 containing 5% Boehringer blocking agent) (Amersham Pharmacia Biotech, Buckinghamshire, UK) and the membrane was washed with washing buffer (0.1% Tween/PBS, pH 7.3). The washing procedure consisted of two quick washes followed by three additional ones: one 15 min and two 5 min washes. Subsequently, the membrane was incubated for 1 h with 2.5 µg mouse anti-Hsp70 monoclonal antibody (SPA-810) (Stressgen Biotechnologies Corporation, Victoria, British Columbia, Canada) in 5 ml blocking solution (0.1% Tween/PBS, pH 7.3 containing 0.5% Boehringer blocking agent). After having performed the above-mentioned washing procedure again, the blots were incubated for 1 h with goat anti-mouse IgG alkaline phosphatase secondary antibody (SAB-101) (Stressgen). The washing procedure was repeated again and the blots were incubated for 5 min in alkaline phosphatase detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 5 mM $MgCl_2.6H_2O$, pH 9.5) containing 2% (v/v) NBT-BCIP (nitroblue

tetrazolium chloride and 5-bromo-4-chloro-3-indoyl-phosphate) (product number 1681451) (Roche Diagnostics GmbH, Mannheim, Germany).

Determination of IL-8 secretion by sandwich ELISA

IL-8 concentrations were assayed using the IL-8 Cytosets™ antibody pair kit containing matched, pre-titered and fully optimized capture and detection antibodies, recombinant standard and streptavidin-horseradish peroxidase (catalog number CHC1304) (Biosource Europe S.A., Nivelles, Belgium). The assay was done according to the manufacturer's specifications.

Statistical analysis

Statistical significance between the mean values of control, heat shocked, butyrate and *S. enteritidis* exposed cells was assessed by one-way analysis of variance (ANOVA) with comparison of means. Differences were considered significant at the $p < 0.05$ level using the Student's T-test.

Results

Inhibition of Salmonella-induced IL-8 secretion by heat shock in Caco-2 cells

Before investigating the modulatory potency of Hsps on IL-8 production in enterocyte-like Caco-2 cells, a chemoattractant that is pivotal to most intestinal inflammations, the pattern of *Salmonella*-induced IL-8 secretion has been determined. Exposure of the cells to 200 bacteria/cell for one hour resulted into a linear time-course IL-8 production (Fig 4.1A, 4.1B). Compared to control cells the secretion is already significantly higher 3 h after infection for both crypt-like (Fig 4.1A) and villus-like cells (Fig 4.1B). When Caco-2 cells were exposed for one hour to graded numbers of bacteria (1, 2, 10, 20, 100, 200 and 1000 bacteria/cell) a significant increase in the secretion of IL-8 was achieved in comparison with control cells. Already on exposure to low numbers of bacteria the levels of IL-8 secretion rapidly increased and the highest levels were measured after incubation with 10 bacteria/cell for both crypt-like (Fig 4.2A) and villus-like cells (Fig 4.2B). While further increasing the numbers of bacteria up to 200 bacteria/cell the levels remained approximately constant. Cell cultures exposed to 1000 bacteria/cell showed distinct indications of cell death that was expressed in a sudden decrease of the levels of IL-8 secretion.

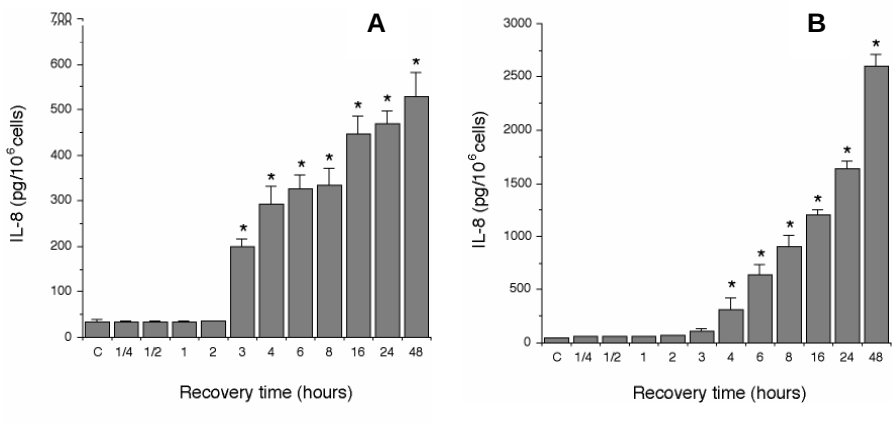

Fig 4.1. IL-8 secretion by Salmonella enteritidis-exposed enterocyte-like Caco-2 cells. *Incubation of the cells was performed with 200 bacteria/cell and the IL-8 secretion was measured at various time points. IL-8 secretion is expressed as pg IL-8/10^6 cells. Salmonella-induced IL-8 secretion was determined using two cell passages and triplicate cultures per passage. Significant differences (*p<0.05) between the IL-8 levels of Salmonella-exposed cells and control Caco-2 cells are indicated. (A) crypt- (B) villus-like Caco-2 cells.*

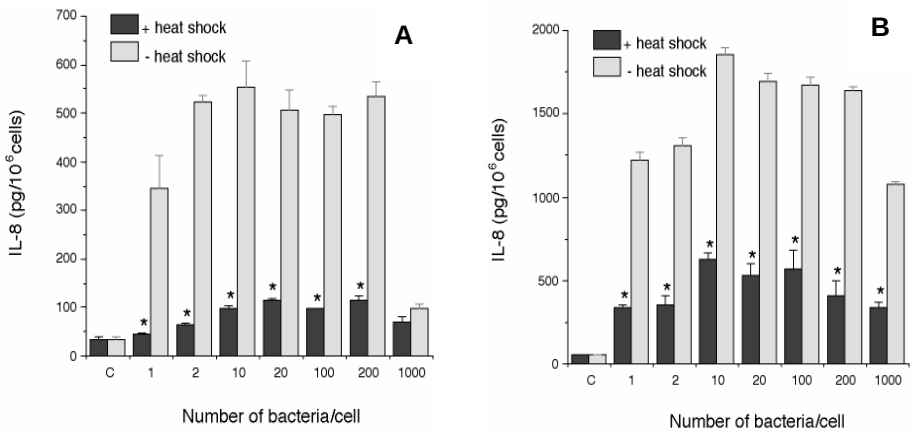

Fig 4.2. Inhibition of Salmonella enteritidis-induced IL-8 secretion in enterocyte-like Caco-2 cells by heat shock treatment. *Control (1 h 37^0C/6 h 37^0C) and heat shocked cells (1 h 42^0C/6 h 37^0C) were exposed to graded numbers of bacteria for 1 h and allowed to recover from bacterial exposure for 24 h. Salmonella-induced IL-8 secretion is expressed as pg IL-8/10^6 cells. Inhibition of Salmonella-induced IL-8 secretion was determined using two cell passages and triplicate cultures per passage. Significant differences (*p<0.05) between the IL-8 levels of heat shocked and control Caco-2 cells are indicated. (A) crypt- (B) villus-like Caco-2 cells.*

When Caco-2 cells received a heat shock, which is known to induce a significant expression of Hsps, prior to incubation with the very same graded numbers of bacteria, this thermal stress attenuated the *Salmonella*-induced IL-8 production in both crypt-like (Fig 4.2A) and villus-like (Fig 4.2B) cells. Exposure of Caco-2 cells to a temperature shock alone did not modify the levels of IL-8 (data not shown). The results clearly demonstrated that the *Salmonella*-induced IL-8 secretion in heat shocked Caco-2 cells had been markedly inhibited.

Induction by butyrate of Hsp70 expression in Caco-2 cells

In order to elucidate whether butyrate induces Hsp70 expression, Caco-2 cells were incubated for 48 h in a range of physiological butyrate concentrations (0.05-20 mM). Compared to control cells, Western blot analysis clearly showed that with increasing butyrate concentrations the expression of Hsp70 in both crypt-like and villus-like Caco-2 cells was significantly enhanced (Fig 4.3). The response was higher in crypt-like than in villus-like cells. A change in the expression could be observed at concentrations as low as 0.5 mM for crypt-like and 5 mM for villus-like cells. At 20 mM the strongest induction was achieved in both cell types. Although this concentration of butyrate induced a significant expression of Hsp70 as can be judged from the immunostaining intensity of these bands in both crypt-like and villus-like cells, the levels of expression did not reach those displayed by heat shocked cells (HS).

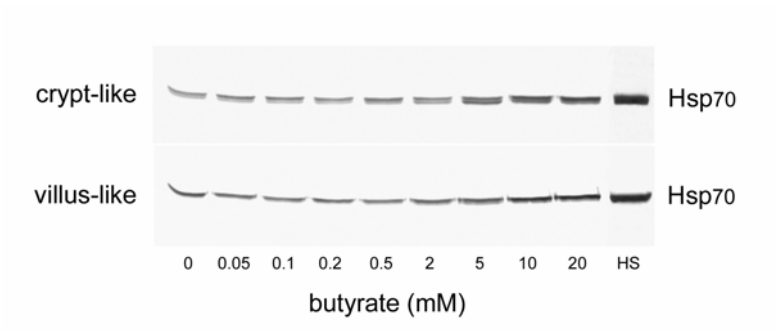

Fig 4.3. Induction of Hsp70 synthesis in enterocyte-like Caco-2 cells during incubation with butyrate. *Cells were incubated for 48 h in a range of physiological butyrate concentrations. HS indicates the level of Hsp70 in cells heat shocked at 42^0C in the absence of butyrate. Subsequently, the cells were processed for Western blotting and immunostaining as described.*

Because the induction of Hsps is generally implicated in cytoprotection during infection and inflammation, the potency of butyrate to induce the expression of Hsp70 in *S. enteritidis* infected Caco-2 cells has been investigated. As demonstrated in Fig 4.4, a dose-dependent Hsp70 expression was exhibited, especially with the crypt-like cells. At 2 mM the levels were already significantly higher and the highest expression was found at 20 mM (Fig 4.4). There was a slight difference among the various butyrate concentrations for villus-like cells. Nevertheless, an increase in Hsp70 expression with 5-20 mM could be observed in these cells (Fig 4.4). In addition, at the highest butyrate concentration the level of Hsp70 expression in *S. enteritidis* infected Caco-2 cells was lower compared to the levels of the thermally induced Hsp70. Since the slots of all gels were loaded with equal amounts of protein (10 µg), the Western blots clearly demonstrate that exposure of the cells to butyrate and bacteria (Fig 4.4) revealed a higher expression than by butyrate alone (Fig 4.3).

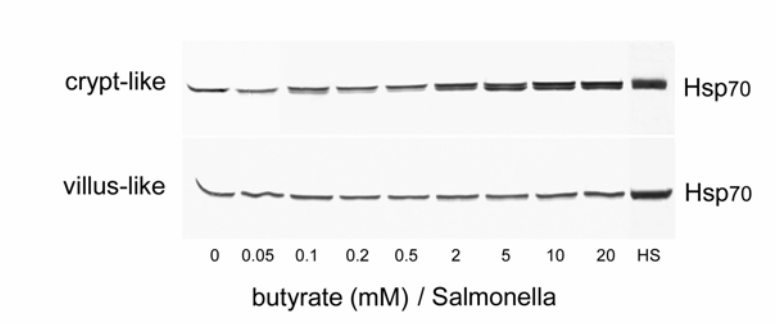

Fig 4.4. Induction of Hsp70 expression in Salmonella enteritidis-exposed enterocyte-like Caco-2 cells after preincubation with butyrate*. After preincubation of the cells for 24 h in a range of physiological butyrate concentrations, cells were exposed to Salmonella (200 bacteria/cell) for 1 h and allowed to recover for 24 h in the very same butyrate concentrations. HS indicates the level of Hsp70 in cells heat shocked at 42^{0}C in the absence of butyrate. After these experimental procedures the cells were processed for Western blotting and immunostaining as described.*

In order to investigate whether thermal stress interfered with the butyrate induced expression of Hsp70, the enterocyte-like Caco-2 cells were heat shocked at 42^{0}C. Taken into account that equal amounts of protein are loaded, the staining intensity of the bands (Fig 4.5) shows that exposure to butyrate and thermal stress enhances further the expression observed by butyrate alone (Fig 4.3). The expression extended to as low as 0.05 mM butyrate, which could not be observed by butyrate exposed cells (Fig 4.3), and exhibited a dose-dependent pattern that

was maximal at 20 mM for both cell types. At this concentration the expression level of Hsp70 was only slightly lower than that induced by heat shock.

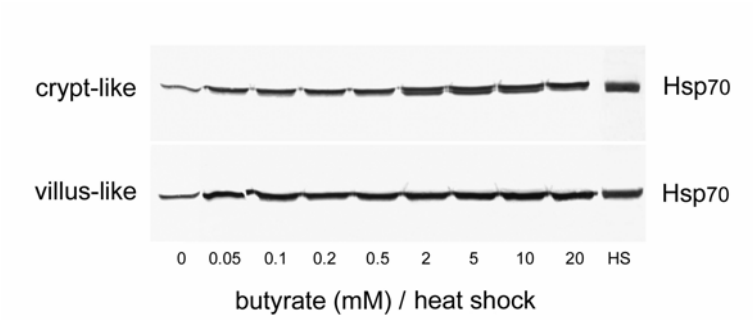

Fig 4.5. Heat shock-induced synthesis of Hsp70 in enterocyte-like Caco-2 cells after preincubation with butyrate. *Cells were incubated for 48 h in a range of physiological butyrate concentrations. The incubation period was interrupted after 24 h and the cells were heat shocked (1 h 42^{0}C/6 h 37^{0}C) in the presence of butyrate. HS indicates the level of Hsp70 in cells heat shocked at 42^{0}C in the absence of butyrate. After these experimental procedures the cells were processed for Western blotting and immunostaining as described.*

Modulation of IL-8 secretion by butyrate in Salmonella-exposed Caco-2 cells

Butyrate is implicated in interfering with chemokine production in intestinal epithelial cells. After having demonstrated the ability of butyrate to induce Hsp70 (Fig 4.3), its effect on IL-8 production in Caco-2 cells was investigated. Incubation of the cells for 48 h in 0.05 to 20 mM butyrate did not induce IL-8 secretion (data not shown). Exposure to *S. enteritidis* (200 bacteria/cell) in the presence of butyrate resulted into a concentration-dependent modulation of IL-8 secretion by both crypt-like (Fig 4.6A) and villus-like (Fig 4.6B) cells. Both inhibitory and stimulatory effects of butyrate on IL-8 secretion could be clearly observed. Low butyrate concentrations of up to 0.2 mM (crypt-like) and 1 mM (villus-like) inhibited the IL-8 secretion achieving the lowest levels at 0.1 mM and 0.5 mM, respectively. The secretion started rising with further increase in butyrate concentrations and reached its highest level at 1 mM and 2 mM for crypt-like and villus-like cells, respectively. Beyond the 1 mM concentration, the IL-8 levels in crypt-like cells exhibited a stable gradual decline with further increase in butyrate concentrations. At 10 and 20 mM the levels of IL-8 did not differ significantly from the levels in

control cells. In villus-like cells, the high butyrate concentrations of 2 to 20 mM maintained high levels of IL-8 production with little or no difference at all in IL-8 levels.

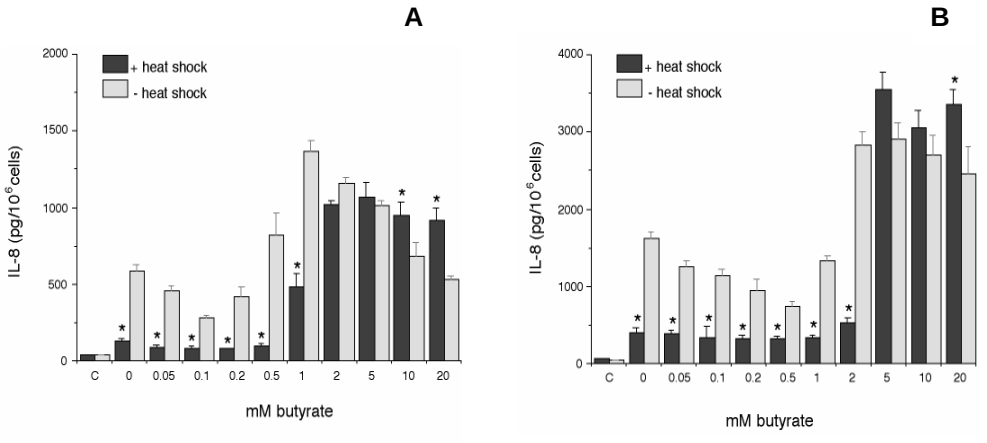

Fig 4.6. Heat shock-induced modulation of Salmonella enteritidis-induced IL-8 secretion in enterocyte-like Caco-2 cells during incubation with butyrate. *Cells were incubated for 48 h in a range of physiological butyrate concentrations. The incubation period was interrupted after 24 h and the cells were heat shocked in the presence of butyrate. Control (1 h 37^{0}C/6 h 37^{0}C) and heat shocked cells (1 h 42^{0}C/6 h 37^{0}C) were then exposed to 200 bacteria/cell for 1 h and allowed to recover from bacterial exposure in the presence of butyrate for the remaining 24 h. Salmonella-induced IL-8 secretion is expressed as pg IL-8/10^6 cells. Inhibition of Salmonella-induced IL-8 secretion was determined using two cell passages and triplicate cultures per passage. Significant differences (*95% confidence intervals) between the IL-8 levels of heat shocked and control Caco-2 cells are indicated. (A) crypt- (B) villus-like Caco-2 cells.*

Effect of thermal stress on IL-8 secretion in Salmonella-exposed Caco-2 cells in the presence of butyrate

To establish whether physiological butyrate concentrations interfere with the anti-inflammatory role of heat stress, Caco-2 cells were incubated for 24 h in 0-20 mM butyrate, heat shocked at 42^{0}C and exposed to *S. enteritidis* (200 bacteria/cell). Subsequently, incubation with the respective butyrate concentrations was continued for 24 h to allow recovery of the cells. Heat shocking the cells in the presence of butyrate prior to exposure to 200 bacteria/cell, inhibited the IL-8 secretion at low butyrate concentrations (0.05-2 mM) and was stimulatory at high ones (5-20 mM) in both crypt-like and villus-like cells. This relationship appeared

to be more pronounced in crypt-like cells (Fig 4.6 A, 4.6B). Whereas thermal stress or butyrate alone did not modify the levels of IL-8 (data not shown), thermal stress induced a significant stimulation of IL-8 secretion by cells incubated in high butyrate concentrations (5-20 mM) (Fig 4.7A, 4.7B).

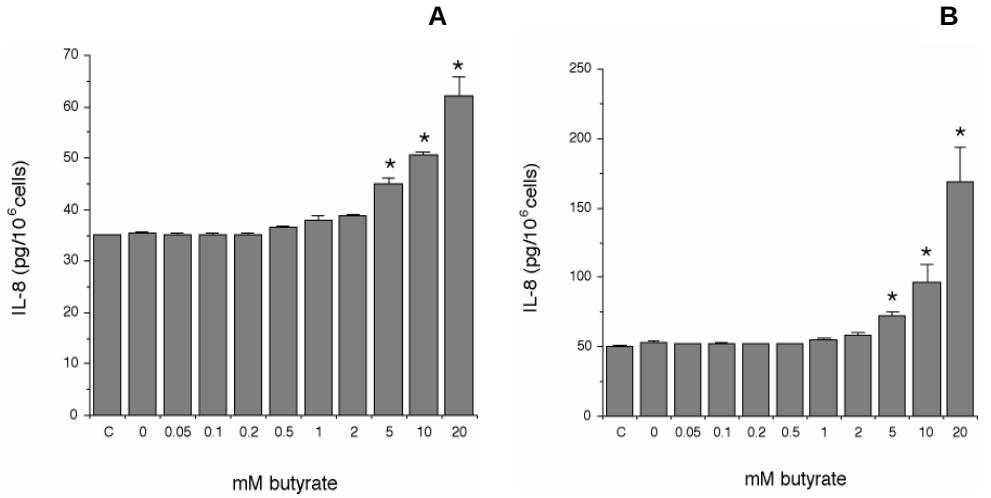

Fig 4.7. Heat shock-induced IL-8 secretion in enterocyte-like Caco-2 cells after preincubation with butyrate. *After exposure to physiological butyrate concentrations (0-20 mM) for 24 h, the control (1 h 37^{0}C) and heat shocked (1 h 42^{0}C) cells were allowed to recover for six hours. During the subsequent heat shock and recovery period, butyrate exposure was continued. IL-8 secretion is expressed as pg IL-8/10^6 cells. Heat shock induced IL-8 secretion was determined using two cell passages and triplicate cultures per passage. Significant differences (*95% confidence intervals) between the levels of IL-8 of heat shocked cells and control Caco-2 cells are indicated. (A) crypt- (B) villus-like Caco-2 cells.*

Discussion

Hsps, which evolutionary have remained remarkably conserved, are a set of proteins involved in coping with chemical and physical stress in all living cells[18,19,28]. Our data presented in this study clearly demonstrated a time-dependent expression of IL-8 secretion (Fig 4.1) and that the presence of high levels of Hsp70 in cells heat shocked at 42^{0}C (Fig 4.3, 4.4, 4.5) inhibited the IL-8 production by enterocyte-like Caco-2 cells exposed to graded numbers of *S. enteritidis* (Fig 4.2). Further, it has been shown that butyrate, dose dependently, induces the expression

of Hsp70 (Fig 4.3) and modulates IL-8 secretion ranging from inhibition to stimulation (Fig 4.6). Following exposure to *S. enteritidis*, the heat shocked cells revealed an augmentation of IL-8 secretion in the presence of butyrate, which was obvious in both crypt-like (2-20 mM) and villus-like (5-20 mM) cells (Fig 4.6). It is clear that these high butyrate concentrations override the inhibitory effect of the heat shock response on IL-8 secretion observed in the absence of butyrate (Fig 4.2). The lower butyrate concentrations of 0.05-0.5 mM for crypt-like and 0.05-2 mM for villus-like cells maintained the inhibitory effect of this short chain fatty acid (Fig 4.6).

The intestinal epithelial cells are capable of synthesizing Hsps that confer protection against thermal stress, infection and inflammation. Protection against inflammation is partly mediated via inhibition of pro-inflammatory cytokine production[20]. Inhibition of IL-8 secretion by Caco-2 cells observed by others is due to prevention of IκB degradation[16], an effect ascribed to Hsp70 in respiratory epithelial cells[22]. Undoubtedly, the thermally induced Hsp70 could account for the IL-8 inhibition observed in this study.

We have demonstrated the potential of butyrate to induce Hsp70 expression (Fig 4.3) and to modulate IL-8 production by enterocyte-like Caco-2 cells (Fig 4.6). The Hsp70 seems to have not taken part in the inhibition of IL-8 production by low butyrate concentrations, because these concentrations did not induce a marked increase in Hsp70 levels (Fig 4.3, 4.4). In addition, the increase in IL-8 production as revealed by high butyrate concentrations, alludes further that butyrate modulates IL-8 secretion via several mechanisms. It is known that butyrate, through histone hyperacetylation, is capable of switching the pattern of chemokine secretion by Caco-2 cells[2,15] on and off via regulation of the nuclear factor kappa B (NF-κB) and the activator protein (AP)-1 transcription factors. By doing so, butyrate can inhibit[14-16] or enhance[2,17] the IL-8 production by Caco-2 cells.

It has been shown that the bacterium *Escherichia coli* induces Hsp70 expression in Caco-2 cells that can be part of the natural mechanism of protection for intestinal epithelial cells in the potentially harmful environment in the intestinal tract[29]. Similarly, the induction of Hsp70 by butyrate observed in our study can be linked up with the IL-8 modulation and may imply a welfare phenomenon for the intestinal epithelium. It is interesting to note that the decline of IL-8 secretion by increasing butyrate concentrations appears to coincide with increasing Hsp70 expression. After reaching its highest levels, IL-8 secretion started to decline with further increase in butyrate concentrations (Fig 4.6A). Though most concentrations retained higher IL-8 levels for villus-like cells (Fig 4.6B), the fall in the IL-8 levels of the crypt-like cells exposed to 20 mM butyrate was such that compared to

control cells these levels became insignificant (Fig 4.6A). This fall was associated with increasing Hsp70 expression for both non-infected (Fig 4.3) and *Salmonella enteritidis* infected cells (Fig 4.4). Based on the anti-inflammatory role of Hsp70, it is reasonable to assume that this IL-8 down-regulation might be mediated, at least in part, through production of Hsp70. The potential of butyrate to induce the production of Hsp25 in rat colon and IEC-18 cell line that is protective against oxidative stress has been demonstrated by others[23]. In consistency, the results of our study clearly demonstrate that butyrate has the potential to induce Hsp70 in enterocyte-like Caco-2 cells.

Induction of the heat shock response in Caco-2 cells exposed to high butyrate concentrations set aside the heat shock inhibitory effect on IL-8 despite inducing Hsp70 (Fig 4.7A, 4.7B). The failure of Hsps to protect intestinal cells against various noxious conditions has been reported. Induction of Hsp60 by thyrotropin-releasing hormone for instance, does not protect colonic mucosa against acetic acid-induced lesions in rats[30]. Depending upon the site of the cells along the gut, the condition of the cell, the luminal environment and the stress causative agent, induced Hsps could or could not be protective. In this study we demonstrated that high butyrate concentrations attenuated the anti-inflammatory effect of the thermally induced Hsp70 in Caco-2 cells incubated with *S. enteritidis* (Fig 4.6).

Our findings that butyrate, dose dependently, modulates IL-8 production by enterocyte-like Caco-2 cells, are supported by the *in vivo* dissemination of intestinal inflammations in the presence of varying butyrate concentrations. It has been reported that high butyrate concentrations are associated with elevated IL-8 levels in the intestinal mucosa of active Crohn's disease patients[7,31]. By contrast, low butyrate concentrations diminish the disease activity[32]. Further, instillation of high concentrations of butyrate into the rectum of healthy mice induces the accumulation of neutrophils and causes inflammatory changes in the colon[4]. Consistent with these facts, our findings also suggest a concentration-dependent butyrate-mediated inhibition or promotion of inflammation via modulation of IL-8 production. The phenomenon implies that low butyrate doses inhibit while higher doses enhance intestinal inflammations. In part, these results may explain the limited therapeutic use of butyrate enemas to patients with Crohn's disease, distal ulcerative colitis and diversion colitis[12,13,32].

The expression of several genes during the inflammatory response including IL-8 biosynthesis is regulated by the NF-κB and the AP-1 transcription factors. The anti-inflammatory role of Hsps stems from the blockade of the NF-κB transcription factors and the modulation of the AP-1 activities. These effects involve inhibition of inhibitory kappa B degradation and the AP-1 inflammatory

responses mediated by p38 of the mitogen activated protein kinase pathway[20]. It is enigmatic the way butyrate induces Hsp production. However, its ability to induce histone hyperacetylation and DNA methylation and the subsequent modulation and binding of transcription factors to their proximal promoter and enhancer elements, could be events of the mechanism. In addition, butyrate can mediate its effects through activation of butyrate response elements, which in turn interfere with the rates of gene transcription[33,34]. Butyrate also induces cellular acidification, which is by itself, a stress condition capable of activating other stress kinases that may culminate into the induction of Hsps[35].

In summary, we have demonstrated the potential of butyrate to induce Hsp70 and the modulation of IL-8 secretion by butyrate and heat shock response in enterocyte-like Caco-2 cells. The heat shock response alone and low butyrate concentrations suppress IL-8 production while higher butyrate concentrations enhance the chemokine production. Because IL-8 is pivotal in the course of inflammation by attracting and activating neutrophils, its inhibition by low concentrations of butyrate or heat shock response contributes to intestinal protection. By contrast, higher butyrate concentrations that enhance IL-8 production suggest a deleterious effect of butyrate. The limitations to the successful therapeutic use of butyrate enemas in colitis may stem from this contrasting butyrate effect.

References

1. Stadnyk AW. 1994. Cytokine production by epithelial cells. *FASEB J* 8: 1041-1047.
2. Fusunyan RD, Quinn JJ, Fujimoto M, MacDermott RP, Sanderson IR. 1999. Butyrate switches the pattern of chemokine secretion by intestinal epithelial cells through histone acetylation. *Mol Med* 5: 631-640.
3. Eckmann L, Fierer J, Kagnoff MF. 1993. Bacterial invasion of epithelial cells induces secretion of the chemoattractant interleukin-8. *Gastroenterology* 104: A694.
4. McCafferty DM, Zeitlin IJ. 1989. Short chain fatty acid-induced colitis in mice. *Int J Tissue React* 11: 165-168.
5. Cummings JH, Branch WJ. 1990. Fermentation and production of short chain fatty acids in human large intestine. In: *Dietary Fiber: Basic and Clinical Aspects*, ed Vahouny GB, Kritchevesky D. Plenum, NY, 131-152.
6. Cummings JH, Pomare EW, Branch WJ, Naylor CPE, MacFarlane GT. 1987. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* 28: 1221-1227.
7. Treem WR, Ahsan N, Shoup M, Hyams J. 1994. Fecal short-chain fatty acids in children with inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 18: 159-164.
8. Bernard JA, Warwick G. 1993. Butyrate rapidly induces growth inhibition and differentiation in HT-29 cells. *Cell Growth Differ* 4: 495-501.

9. Frankel WL, Zhang W, Singh A, Klurfeld DM, Don S, Sakata T, Modlin I., Rombeau JL. 1994. Mediation of the trophic effects of short-chain fatty acids on the rat jejunum and colon. *Gastroenterology* 106: 375-380.
10. Roediger WEW. 1980. The colonic epithelium in ulcerative colitis: an energy-deficiency disease? *Lancet* 2: 712-715.
11. Scheppach W, Sommer H, Kirchner T, Paganelli GM, Bartram P, Christl S, Richter F, Dusel G, Kasper H. 1992. Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. *Gastroenterology* 103: 51-56.
12. Harig JM, Soergel KH, Komorowski RA, Wood CM. 1989. Treatment of diversion colitis with short chain fatty acid irrigation. *N Engl J Med* 320: 23-28.
13. Sanderson IR. 1997. Diet and gut inflammation. *Curr Opin Gastroenterol* 13: 518-524.
14. Huang N, Katz JP, Martin DR, Wu GD. 1997. Inhibition of IL-8 gene expression in Caco-2 cells by compounds which induce histone hyperacetylation. *Cytokine* 9: 27-36.
15. Andoh A, Fujiyama Y, Hata K, Araki Y, Takaya H, Shimada M, Bamba T. 1999. Counter-regulatory effect of sodium butyrate on tumuor necrosis factor-alpha (TNF-α)-induced complement C3 and factor B biosynthesis in human intestinal epithelial cells. *Clin Exp Immunol* 118: 23-29.
16. Wu GD, Huang N, Wen X, Keilbaugh SA, Yang H. 1999. High-level expression of IκB-β in the surface epithelium of the colon: *in vitro* evidence for an immunomodulatory role. *J Leukoc Biol* 66: 1049-1056.
17. Fusunyan RD, Quinn JJ, Ohno Y, MacDermott RP, Sanderson IR. 1998. Butyrate enhances interleukin (IL)-8 secretion by intestinal epithelial cells in response to IL-1β and lipopolysaccharide. *Pediatr Res* 43: 84-90.
18. Wu C. 1995. Heat shock transcription factors: Structure and regulation. *Annu Rev Cell Dev Biol* 11: 441-469.
19. Ovelgönne JH, Koninkx JFJG, Pusztai A, Bardocz S, Kok W, Ewen SW, Hendriks HG, van Dijk JE. 2000. Decreased levels of heat shock proteins in gut epithelial cells after exposure to plant lectins. *Gut* 46: 679-687.
20. Malago JJ, Koninkx JFJG, van Dijk JE. 2002. The heat shock response and cytoprotection of the intestinal epithelium. *Cell Stress Chaperones* 7: 191-199.
21. Tsuruma T, Yagihashi A, Matsuno T, Zou XM, Asanuma K, Sasaki K, Hirata K. 1996. The heat-shock protein 70 family reduces ischemia/reperfusion injury in the small intestine. *Transplant Proc* 28: 2629-2630.
22. Yoo C, Lee S, Lee C, Kim YW, Han SK, Shim Y. 2000. Anti-inflammatory effect of heat shock protein induction is related to stabilization of IκBα through preventing IκB kinase activation in respiratory epithelial cells. *J Immunol* 164: 5416-5423.
23. Ren H, Musch MW, Kojima K, Boone D, Ma A. 2001. Short-chain fatty acids induce intestinal epithelial heat shock protein 25 expression in rats and IEC 18 cells. *Gastroenterology* 121: 631-639.
24. Pinto M, Robine-Leon S, Appay MD, Kedinger M, Triadou N, Dussaulx E, Lacroix B, Simon-Assmann P, Haffen K, Fogh J, Zweibaum A. 1983. Enterocyte-like differentation and polarization of the human colon carcinoma Caco-2 cell line in culture. *Biol Cell* 47: 323-330.
25. Koninkx JFJG, Hendriks HGCJM, van Rossum JMA, van den Ingh TSGAM, Mouwen JMVM. 1992. Interaction of legume lectins with the cellular metabolism of differentiated Caco-2 cells. *Gastroenterology* 102: 1516-1523.
26. Koninkx JFJG. 1995. Enterocyte-like Caco-2 cells as a tool to study lectin interaction. In: *Lectins: Biomedical Perspectives*, ed Pusztai A, Bardocz S. Taylor and Francis, London, 81-101.

27. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic acid. *Anal Biochem* 159: 76-85.
28. Welch WJ. 1992. Mammalian stress response: Cell physiology, structure/function of stress proteins, and implications for medicine and disease. *Physiol Rev* 72: 1063-1081.
29. Deitch EA, Beck SC, Cruz NC, Maio AD. 1995. Induction of heat shock gene expression in colonic epithelial cells after incubation with *Escherichia coli* or endotoxin. *Crit Care Med* 23: 1371-1376.
30. Iwabuchi A, Otaka M, Otani S, Jin M, Okuyama A, Itoh S, Sasahara H, Odashima M, Ivan P, Itoh H, Tashima Y, Masamune O, Watanabe S. 2000. Specific preinduction of 60-kDa heat shock protein (chaperonin homolog) by TRH does not protect colonic mucosa against acetic acid-induced lesions in rats. *Dig Dis Sci* 45: 1480-1489.
31. Dabard J, Hudault S, Saby MA, Gabilan JC, Szylit O, Raibaud P. 1987. Production of butyric acid in human premature baby suffering from necrotizing enterocolitis. Proceedings of the 9th International Symposium on Gnotobiology, Versailles, 90-95.
32. O'Morain C, Segal AW, Levi AJ. 1982. Elemental diet as primary treatment of acute Crohn's disease: A controlled trial. *BMJ* 288: 1859-1862.
33. Lallemand F, Corilleau D, Sabbah M, Reduilh G, Mestger J. 1996. Direct inhibition of the expression of cyclin D1 gene by sodium butyrate. *Biochem Biophys Res Commun* 229: 163-169.
34. Nakano K, Mizuno T, Sowa Y, Orita T, Yoshino Y, Fujita T, Ohtani-Fujita N, Matsukawa Y, Tokino Y, Yamagishi H, Oka T, Nomura H, Sakai T. 1997. Butyrate activates the Waf1/Cip1 gene promoter through Sp1 sits in a p53-negative human colon cancer cell line. *J Biol Chem* 272: 22199-22206.
35. Amemiya M, Yamaji Y, Cano A, Moe OW, Alpern RJ. 1995. Acid incubation increases NHE-3 mRNA abundance in OKP cells. *Am J Physiol* 269: C126-C133.

5

EXPRESSION LEVELS OF HEAT SHOCK PROTEINS IN ENTEROCYTE-LIKE CACO-2 CELLS AFTER EXPOSURE TO SALMONELLA ENTERITIDIS

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Abstract

The enterocytes of the small intestine are occasionally exposed to pathogenic bacteria, such as *Salmonella enteritidis 857*, an etiologic agent of intestinal infections in human. The expression of the heat shock response by enterocytes may be part of a protective mechanism developed against pathogenic bacteria in the intestinal lumen. We aimed at investigating whether *S. enteritidis 857* is able to induce a heat shock response in crypt- and villus-like Caco-2 cells and at establishing the extent of the induction. To establish whether *S. enteritidis 857* interfered with the integrity of the cell monolayer, the transepithelial electrical resistance (TEER) of filter-grown, differentiated (villus-like) Caco-2 cells was measured. We clearly observed damage to the integrity of the cell monolayer by measuring the TEER. The stress response was screened in both crypt- and villus-like Caco-2 cells exposed to heat (40-43°C) or to graded numbers (10^1-10^8) of bacteria and in villus-like cells exposed to *S. enteritidis 857* endotoxin. Expression of the heat shock proteins Hsps70 and Hsp90 was analyzed by polyacrylamide gel electrophoresis and immunoblotting with monoclonal antibodies. Exposure to heat or *Salmonella* resulted in increased levels of Hsp70 and Hsp90 in a temperature-effect or *Salmonella*-dose relationship, respectively. Incubation of Caco-2 cells with *S. enteritidis 857* endotoxin did not induce heat shock gene expression. We conclude that *S. enteritidis 857* significantly increases the levels of stress proteins in enterocyte-like Caco-2 cells. However, our data on TEER clearly indicate that this increase is insufficient to protect the cells.

Introduction

Living cells exhibit a universal response to adverse changes in their environment, which is commonly known as the heat shock or stress response[1-3]. Apparently a defensive mechanism[4], the transient heat shock response is a complex phenomenon that is rapidly induced and protects the cells from irreversible injury by stabilizing the synthetic and metabolic activities in the cell. The most obvious characteristics of the stress response are an enhanced synthesis of heat shock proteins (Hsps), commonly known as molecular chaperones, and a concomitant inhibition of overall protein synthesis[5-7]. The Hsps were originally discovered in isolated heat-shocked *Drosophila melanogaster* salivary glands, where their appearance coincides with chromosomal puffs[8]. These chromosomal puffs represent specific transcription sites for synthesis of Hsps[5,6,8]. In mammalian cells, induction of Hsps is considered to be regulated mainly at the transcriptional level by activity of a specific heat shock transcription factor[9]. In addition to transcriptional regulation, translational regulation has been described in insect cells[10], and there is also some evidence of this type of regulation in mammalian cells[11].

There is sufficient evidence to link the stress response to a consequent decrease in cellular sensitivity to stress. It has been clearly demonstrated that thermotolerance is conferred by increased levels of Hsps. This has been observed under conditions in which Hsps are induced by environmental stress[12] as well as by transfection of Hsp genes[13,14].

A large and increasing body of information indicates that the heat shock response is elicited not only by hyperthermia but also by a wide variety of other stimuli[2,15]. Exposure of cells to such stressors, including oxidizing agents, anoxia, heavy metals, vitamin B_6, sulfhydryl agents, and ethanol, results in a virtual shutdown of normal cellular protein synthesis, paralleled by a shift to high levels of Hsp synthesis[6,7]. In the unstressed cells, constitutive levels of Hsps are involved in the restoration of unfolded or aggregated polypeptides to their native conformations, the proteolysis of proteins too damaged to refold, the assembly of proteins, and the translocation of proteins across membranes[3,16]. Because of these functions, Hsps are called molecular chaperones. This function of the Hsps enables the cell to survive during stress and promotes the resumption of normal cellular activities in the recovery period after stress. For a number of disease states, including tissue necrosis, viral infection, inflammation and cancer, abnormally high expression levels of Hsps have been observed[2,16]. As a consequence of dietary intake, gut epithelial cells are regularly exposed to high levels of potentially harmful substances of dietary origin such as lectins and bacteria.

Cell cultures displaying enterocyte-like differentiation are suitable models for studying the mechanisms underlying the proliferation and differentiation of intestinal epithelial cells at the cellular level[17-19]. The human colon carcinoma cell line Caco-2, which is phenotypically similar to human small intestinal enterocytes, is a particularly important *in vitro* model. At late confluency, these cells display differentiation characteristics of small intestinal enterocytes, both structurally and functionally[18,20].

Through their adhesins, *Salmonella* species are capable of binding and invading mucosal barriers[21,22]. This behavior is also exhibited with mucosal models consisting of the polarized epithelial Caco-2 cell line[23,24]. Infection of this cell line with *S. typhimurium* has been shown to cause structural lesions at the apical membrane of the cells and to elicit severe disruptions in the integrity of the epithelial monolayer[25,26]. Because *S. enteritidis* 857, a cause of food poisoning, is known as an etiologic agent of intestinal infections in humans, this strain[24] has been used in our experiments.

The experiments of this study were designed to investigate whether bacteria induce changes in the heat shock response of enterocyte-like Caco-2 cells. Both 5-day-old, undifferentiated Caco-2 cells, the *in vitro* counterpart of small intestinal crypt cells, and 19-day-old, differentiated ones, the *in vitro* counterpart of small intestinal villus cells, have been used in this investigation. In particular, we have examined the effect of *S. enteritidis* 857 on the expression of Hsp70 and Hsp90 and established the extent of this expression.

Materials and Methods

Cell culture

Caco-2 cells (American Type Culture Collection) were routinely grown and maintained in Dulbecco modified Eagle medium (DMEM) (Flow Laboratories, Amsterdam, The Netherlands) and cultured at 37^0C in a humified atmosphere of 5% (v/v) CO_2 in air. This medium was supplemented with 1% nonessential amino acids, 50 µg ml^{-1} gentamicin, 10 mM sodium bicarbonate, 25 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethane-sulfonic acid (4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid), (all Flow) and 20% (v/v) fetal calf serum (FCS) (Ritmeester BV, Utrecht, The Netherlands). Supplemented DMEM devoid of gentamicin and FCS was referred to as plain DMEM. Cells were seeded at 40 000 cells cm^{-2} in tissue culture flasks (25 cm^2) and in 12-well tissue culture plates (4 cm^2) (both Greiner, Alphen a/d Rijn, The Netherlands). Cells grown on Transwell, polycarbonate filter inserts of 12-well tissue culture plates (0.4-µm pore size)

(Costar Europe Ltd, Badhoevedorp, The Netherlands), were seeded at 60 000 cells cm^{-2}.

The Caco-2 cells were cultured for 5 days to achieve undifferentiated cells (the *in vitro* counterpart of crypt cells) or 19 days to achieve fully differentiated cell populations (the *in vitro* counterpart of villus cells). The cell culture medium was changed 3 times a week.

Heat shock

The duration of the heat shock (40^0C, 41^0C and 42^0C) was 1 h, whereas the recovery period at 37^0C lasted 6 h. During the entire heat shock procedure, supplemented cell culture medium was used. Heat shocks were applied by placing the tissue culture flasks in water heated by a circulating thermostat DC10 (Haake, Karlsruhe, Germany). This thermostat maintained stable temperatures within 0.02^0C. Under these conditions, temperature equilibration of the cell monolayers took about 30 s.

Exposure of Caco-2 cells to S. enteritidis 857 and endotoxins

A single colony of *S. enteritidis* 857^{24} grown on Luria-Bertani (LB) agar was inoculated into 10 ml LB broth and grown overnight (16 h) at 37^0C with shaking (200 rpm). From this culture, 300 µl of the bacterial suspension was inoculated into 30 ml LB broth and incubated with shaking (200 rpm) at 37^0C for 2 h to obtain logarithmically growing bacteria. Subsequently, bacteria were collected by centrifugation (10 min at 1500*g*; 22^0C) and finally suspended in 30 ml plain DMEM. The suspension was divided in three aliquots of 10 ml each. One of these aliquots was centrifuged (10 min at 1500*g*; 22^0C), supernatant was discarded, and bacteria were killed by suspending them in 96% ethanol for 30 min. They were then washed twice in plain DMEM and finally suspended in 10 ml plain DMEM. To the second aliquot, chloramphenicol (20 µg ml^{-1}) was added just before infecting the cells. The Caco-2 cells were washed twice with 5 ml plain DMEM prior to bacterial infection.

To establish whether bacteria themselves show a heat shock response, 20 µl of the bacterial suspension grown overnight was inoculated into two tubes containing 2 ml LB broth and incubated at 37^0C or 42^0C for 60 min. After 2 washes with 5 ml 0.01 M phosphate-buffered saline (PBS) (0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, 0.9% (w/v) NaCl), pH 7.3, the bacterial pellets were boiled for 10 min in 1 ml sample buffer (see Western Blot Analysis section).

Because incubation of Caco-2 cells with *S. enteritidis* 857 was done in plain DMEM, the amount of FCS was gradually reduced and replaced by Ultroser

G, a serum substitute (Gibco Europe, Hoofddorp, The Netherlands). This reduction was accomplished by replacing the cell culture medium containing 20% FCS with medium containing 10% FCS and 1% Ultroser G on day 3 (for crypt-like Caco-2) and 17 (for villus-like Caco-2). This medium was then substituted by a medium containing 1% FCS and 2% Ultroser G 1 day later (days 4 and 18). On day 5 and 19 Caco-2 cells were incubated in quadruplicate with *S. enteritidis* 857 (10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 bacteria ml^{-1}) for 1 h at 37^0C in 1 ml plain DMEM. Monolayers of 19-day-old cells were also incubated with various amounts of *S. enteritidis* (LPS^{Se}) and *Escherichia coli* 0111:B4 (LPS^{Ec}) endotoxins (20 and 100 µg ml^{-1}) under the same conditions. After bacterial or endotoxin exposure, the cell monolayer was washed twice with 1 ml gentamicin containing plain DMEM, and incubation was continued for a further 6 h (recovery period) using the same medium.

Transepithelial electrical resistance of Caco-2 cell monolayers after exposure to bacteria

Mucosal integrity of Caco-2 cells seeded (60 000 cells cm^{-2}) on polycarbonate, 0.4-µm-pore size tissue culture inserts in 12-well plates (insert growth area, 1 cm^2) was verified by measuring the transepithelial electrical resistance (TEER) with a Millicell-ERS V Ω^{-1} meter (Millipore Corporation, Bedford, MA, USA). This device contained a pair of chopstick electrodes, which facilitated the measurements. Cell monolayers were used for experiments 19 days after seeding, the mean TEER ± SD for control cells being 205 ± 12 Ω cm^{-2} after subtracting the resistance of blank filters.

For exposure studies, filter-grown monolayers (apical volume, 600 µl; basolateral volume, 1500 µl) were first equilibrated with plain DMEM for 2 h under cell culture conditions. Subsequently, 60 µl of apical DMEM was removed and replaced by the same volume of DMEM containing bacteria. After exposure to *S. enteritidis 857* (10^4, 10^5, 10^6, 10^7 and 10^8 bacteria ml^{-1}) for 1 h in quadruplicate, monolayers were washed twice with 1 ml gentamicin containing plain DMEM, and incubation was continued. Changes in TEER were measured 1, 2, 3, 4, 5, 6, 12, 24, and 48 h after the washing step.

Western blot analysis

After the recovery period the cells exposed to bacteria (4 cm^2), as well as the heat-shocked cells (25 cm^2), were washed twice at 4^0C with 1 or 5 ml PBS. Cells of 1 well or flask were scraped off into 1 or 5 ml distilled water, and cell

scrapings were sonicated at 0^0C for 30 s at an amplitude of 24 µm with an MSE Soniprep 150 (Beun de Ronde BV, Abcoude, The Netherlands). The protein content of the resulting sonicates was determined[27]. An equal volume of sample buffer (twice the strength) was added to the protein samples, after which they were boiled for 10 min. Subsequently, the slots of the gel were loaded with equal amounts of proteins (12 µg) and the proteins separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis on 10% gels. The sample buffer (normal strength) consisted of 125 mM Tris-(hydroxymethyl)aminomethane-HCl, 4% SDS, 10% ß-mercaptoethanol, 20% glycerol and 0.0015% bromophenolblue, pH 6.8. Subsequently, proteins were transferred to Immobilon-P polyvinylidene difluoride membrane, according to the recommendations of the manufacturer (Millipore). Hsp70 (number SPA-810) and Hsp90 (number SPA-830) were detected using monoclonal antibodies purchased from Stressgen Biotechnologies Corporation (Victoria, British Columbia, Canada) and a peroxidase-coupled detection system (Bio-Rad, Hercules, CA, USA). Quantification of the stained blots was performed on a Bio-Rad GS700 imaging densitometer.

To assess statistical significance between the quantified staining intensities of control Caco-2 cells and cells exposed to *S. enteritidis* 857, an analysis of variance and comparison of means was used. Statistical significance was accepted at the $P<0.05$ level.

Results

S. enteritidis 857 induced alterations in TEER

To investigate whether *S. enteritidis 857* interfered with the mucosal integrity of the Caco-2 cell monolayer, filter-grown, differentiated cells were incubated with graded numbers of bacteria in plain DMEM for 1 hour. Compared with control cells, *S. enteritidis* 857-exposed, filter-grown, differentiated Caco-2 cells clearly showed changes in TEER. There was little (Fig 5.1B) or no change at all (Fig 5.1A) in TEER when cells were incubated with small numbers of bacteria (10^4 and 10^5). In contrast, exposure of the cells to further increasing numbers of bacteria (10^6, 10^7 and 10^8) revealed a significant decrease in TEER (Fig 5.1B). A full recovery of the TEER to control values, setting in 12 h after the washing step with gentamicin, was observed when Caco-2 cells had been exposed to 10^6 bacteria. In contrast, there was no recovery at all after incubation with 10^7 and 10^8 bacteria. In this case, the values of the TEER were approaching the resistance of a blank filter (Fig 5.1B). Microscopic examination clearly showed that the cell monolayer with this low TEER value had been damaged severely.

Heat shock induced synthesis of Hsp70 and Hsp90 in Caco-2 cells

Before investigating the heat shock gene expression by bacteria in enterocyte-like Caco-2 cells, the capability of the cells to respond to heat shock was determined. Exposure of 19-day-old fully differentiated Caco-2 cells to a temperature shock was found to induce the expression of Hsps.

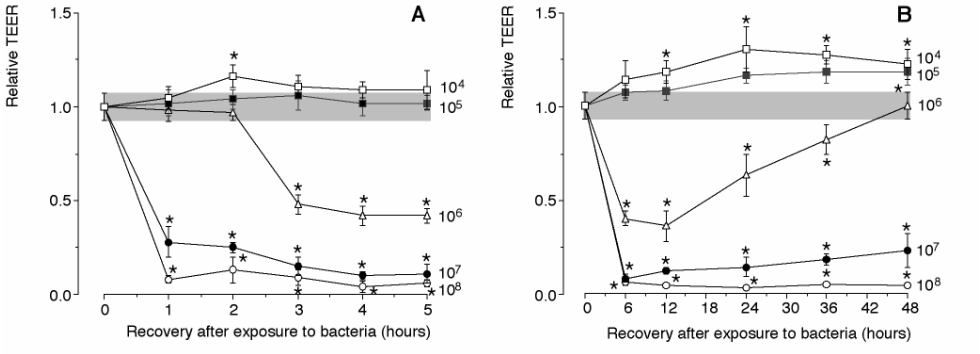

Fig 5.1. Dose dependency of Salmonella enteritidis 857 induced changes of the transepithelial electrical resistance (TEER) in monolayers of differentiated Caco-2 cells after apical exposure. *Caco-2 cells were grown on tissue culture inserts (pore size, 0.4 µm; growth area, 1 cm^2). Before the experiments, filter-grown monolayers were first equilibrated with plain Dulbecco modified Eagle medium (DMEM) for 2 h and subsequently incubated at 37^0C for 1 h with S. enteritidis 857 (10^4, 10^5, 10^6, 10^7, and 10^8 bacteria ml^{-1}). After 2 washes with 1 ml gentamicin containing plain DMEM, the time course of the changes in TEER was measured. The results are expressed as the mean relative TEER ± SD. The dotted area represents the mean relative TEER ± SD of cell cultures not exposed to bacteria. Significant differences ($P < 0.05$) between the relative TEER levels of bacteria-exposed and control cells are indicated by an asterisk. The relative levels were established using 2 cell passages and triplicate cultures per passage. Fig 5.1A shows in detail the data for the initial 5 h after exposure, whereas Fig 5.1B demonstrates the effect up to 48 h.*

A temperature-effect relationship could be clearly observed for both Hsps (Fig 5.2A and 5.2B). Compared with control cells maintained at 37^0C, which expressed constitutive levels of Hsp70 and Hsp90, the levels of these Hsps at 42^0C in 19-day-old, villus-like cells had increased significantly. A 1.8-fold increase was achieved in the level of expression for Hsp90, whereas the expression level for Hsp70 increased 3.7-fold. Cell cultures exposed to 43^0C showed distinct indications of cell death, and the level of heat shock response was even lower than at 40^0C (data not shown).

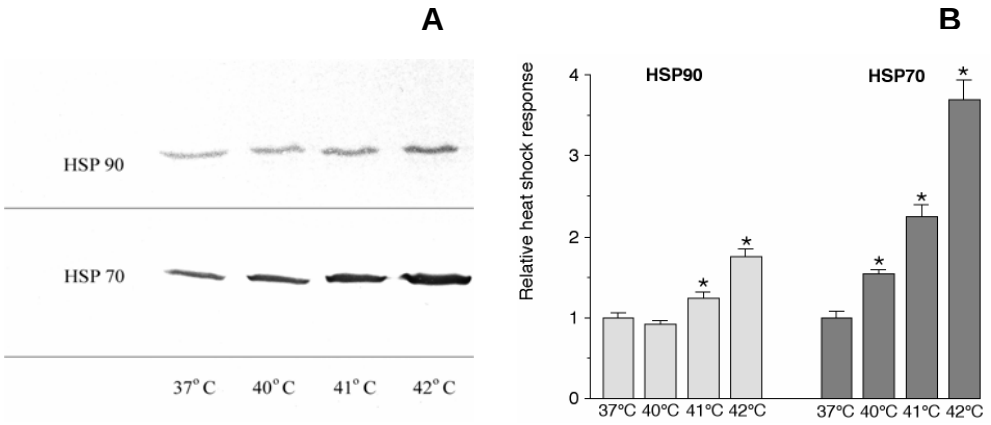

Fig 5.2. Induction of Hsp70 and Hsp90 in differentiated Caco-2 cells. *Hsp70 and Hsp90 were induced by exposure to 40^{0}C, 41^{0}C and 43^{0}C for 1 h, followed by recovery at 37^{0}C for 6 h. (A) After these experimental procedures, the cells were processed for Western blotting and immunostaining. (B) Quantification of the Western blots revealed significant differences ($P < 0.05$) (indicated by an asterisk) between the relative levels of Hsps of heat-shock exposed and control cells for 2 sets of experiments, each done in triplicate.*

Also in 5-day-old, crypt-like cells, the level of expression for both Hsps increased significantly. Compared with 19-day-old cells the levels in these cells had increased 1.6-fold for Hsp90 and 1.5-fold for Hsp70 (Fig 5.2A and 5.2B). For comparison purposes, heat-shocked cells (1 h exposure at 42^{0}C; 6 h recovery at 37^{0}C) were used as positive controls and those maintained for 7 h at 37^{0}C as negative ones.

It was not possible to detect bacterial Hsp70 and Hsp90 by using the antibodies used in this study. This confirmed their suitability for the present experiments (data not shown).

Hsp response in S. enteritidis 857 exposed crypt-like and villus-like Caco-2 cells

To investigate whether *S. enteritidis* 857 was able to induce the heat shock response in enterocyte-like Caco-2 cells, cells were incubated with bacteria in plain DMEM for 1 h at 37^{0}C. When compared with control, 5-day-old, undifferentiated, crypt-like Caco-2 cells, a significant increase in the level of Hsp90 was achieved after exposure to bacteria. After incubation with 10^5, 10^6, 10^7 and 10^8 bacteria, the level of expression of Hsp90 approached the expression of this protein after heat shock at 42^{0}C (Fig 5.3A). The expression level of Hsp70 in crypt-like Caco-2 cells

also significantly increased after incubation with 10^4, 10^5, 10^6, 10^7 and 10^8 *S. enteritidis* 857 bacteria. When cells were exposed to 10^7 or 10^8 bacteria, a 1.5-fold increase over the control level was observed. This level of Hsp70 after bacterial exposure matched the level in heat-shocked (42^0C) cells (Fig 5.3B).

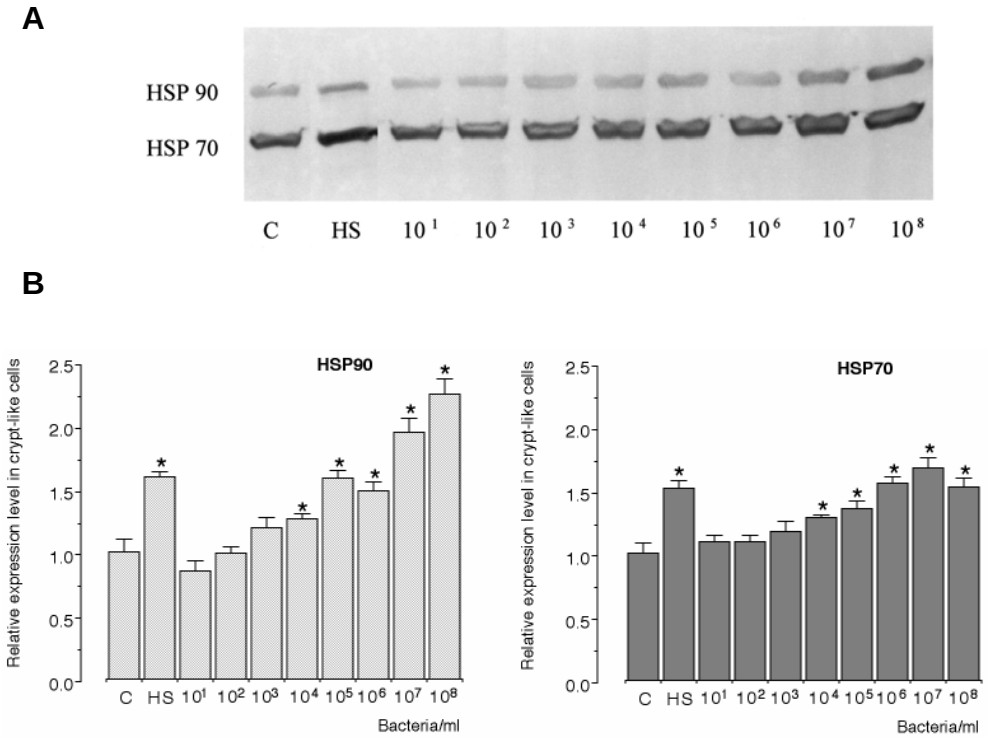

Fig 5.3. Expression levels of Hsp70 and Hsp90 in 5-day-old, undifferentiated Caco-2 cells after exposure to Salmonella enteritidis 857. *Cells were exposed for 1 h to 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 or 10^8 bacteria ml^{-1} plain* ***Dulbecco modified Eagle medium (DMEM)****. (A) After 6 h of recovery in plain DMEM containing gentamicin, cells were processed for Western blotting and immunostaining. (B) Quantification of the Western blots revealed significant differences ($P < 0.05$) (indicated by an asterisk) between the relative levels of heat shock proteins (Hsps) of Salmonella enteritidis 857-exposed and control cells (B). The relative levels of Hsps were established using 2 cell passages and triplicate cultures per passage. The results are expressed as the relative amount of Hsp ± SD. (C: levels of Hsp70 and Hsp90 in control Caco-2 cells; HS: levels of Hsp70 and Hsp90 in heat-shocked cells (1 h at 42^0C; 6 h recovery at 37^0C)).*

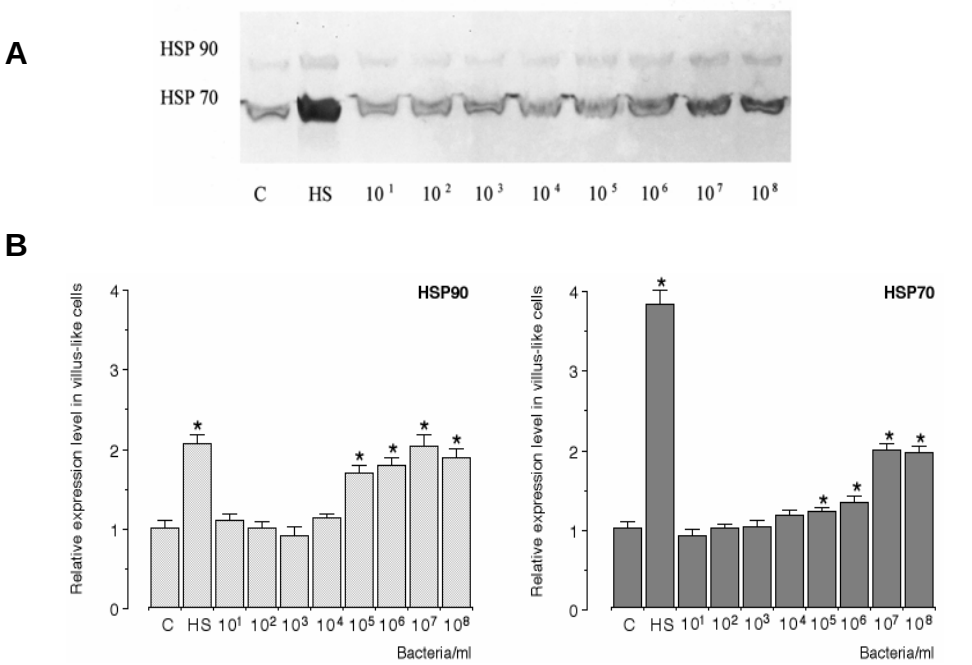

Fig 5.4. Expression levels of Hsp70 and Hsp90 in 19-day-old, differentiated Caco-2 cells after exposure to Salmonella enteritidis. *Cells were exposed for 1 h to 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 or 10^8 bacteria ml^{-1} plain Dulbecco modified Eagle medium (DMEM). (A) After 6 h of recovery in plain DMEM containing gentamicin, cells were processed for Western blotting and immunostaining. (B) Quantification of the Western blots revealed significant differences ($P < 0.05$) (indicated by an asterisk) between the relative levels of heat shock proteins (Hsps) of Salmonella enteritidis 857-exposed and control cells (B). The relative levels of Hsps were established using 2 cell passages and triplicate cultures per passage. The results are expressed as the relative amount of Hsp ± SD. (C: levels of Hsp70 and Hsp90 in control Caco-2 cells; HS: levels of Hsp70 and Hsp90 in heat-shocked cells (1 h at 42^0C; 6 h recovery at 37^0C)).*

Exposure of 19-day-old, differentiated, villus-like Caco-2 cells to *S. enteritidis* 857 was found to induce the expression of both Hsp70 and Hsp90 (Fig 5.4A). With respect to Hsp90 10^7 or 10^8 *S. enteritidis* 857 bacteria appeared to be needed to bring about the same 1.7-fold increase in the heat shock response, which is inducible at 42^0C (Fig 5.4B). However, compared with the 3.7-fold increase in the level of expression of Hsp70 in heat-shocked (42^0C) cells, the heat shock response of this protein after exposure to bacteria was only moderate. In this case,

10^7 or 10^8 bacteria were needed to achieve a 1.5-fold increase over the control levels in 19-day-old, villus-like Caco-2 cells (Fig 5.4B).

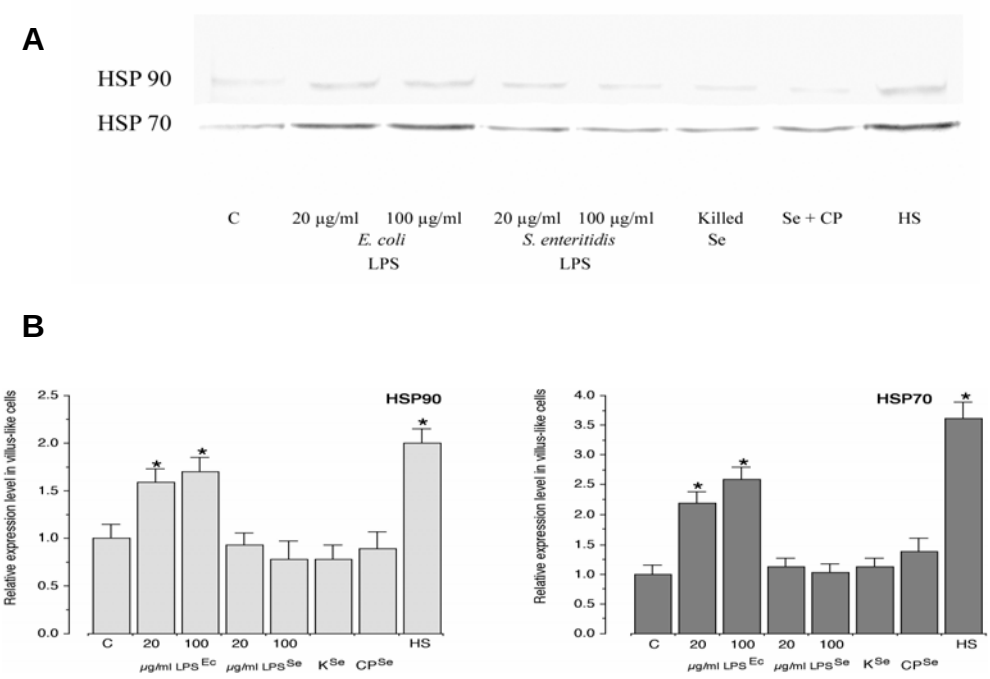

Fig 5.5. Expression levels of Hsp70 and Hsp90 in 19-day-old, differentiated Caco-2 cells after exposure to Salmonella enteritidis 857 or Escherichia coli endotoxins, killed or S. enteritidis incubated with chloramphenicol. *Cells were exposed to S. enteritidis 857 or E. coli 0111:B4 lipopolysaccharides (LPS) at 20 or 100 µg ml^{-1} plain Dulbecco modified Eagle medium (DMEM) for 1 h. In another experiment, cells were exposed to either killed or live S. enteritidis incubated with 20 µg ml^{-1} of chloramphenicol at 10^8 bacteria ml^{-1} plain DMEM for 1 h. (A) After 6 h of recovery in plain DMEM containing gentamicin, cells were processed for Western blotting and immunostaining. (B) Quantification of the Western blots revealed significant differences ($P < 0.05$) (indicated by an asterisk) between the relative levels of Hsps of heat shock or E. coli LPS-exposed cells and control cells. The relative levels of Hsps were established using 2 cell passages and triplicate cultures per passage. The results are expressed as the relative amount of Hsp ± SD. (C: levels of Hsp70 and Hsp90 in control Caco-2 cells; HS: levels of Hsp70 and Hsp90 in heat shocked cells (1 h at 42^0C; 6 h recovery at 37^0C); LPS^{Ec}/LPS^{Se}: Escherichia coli/S. enteritidis lipopolysaccharide; CP^{Se}/Se+CP: S. enteritidis incubated with chloramphenicol; K^{Se}: killed S. enteritidis).*

No induction at all of Hsp70 and Hsp90 was found in Caco-2 cells incubated with *S. enteritidis* 857 (10^8) in the presence of chloramphenicol, killed bacteria or its endotoxin (20 or 100 μg ml^{-1}). In these cases the levels of expression of these proteins were similar to their expression in control cells. However, exposure to *E. coli* 0111:B4 endotoxin (20 or 100 μg ml^{-1}) induced a significant expression of these proteins (Fig 5.5A and 5.5B).

Discussion

The highly conserved set of stress proteins is involved in coping with chemical and physical stress in all living cells[1-3]. The data presented in this investigation clearly demonstrate that *S. enteritidis* 857 increases the levels of Hsp70 and Hsp90 in these cells. In crypt-like and villus-like cells, the Hsp content increases significantly on exposure to bacteria.

Binding of bacteria to cells lining the intestine induces a decrease in the number of microvilli covering the cells, and simultaneously with this change, ruffle formation takes place[28]. Recent studies with *Salmonella* species have revealed that membrane ruffling, which is associated with the site of entry of the bacteria, occurs in the apical membrane during bacterial binding and invasion[29,30]. At these sites, a rearrangement of actin filaments take place. Both *Salmonella*-induced ruffles and the subsequent entry of bacteria, are sensitive to inhibitors of actin filament polymerization[31]. Actin disruption of polarized Caco-2 cells can augment internalization of bacteria, in which exposure of the lateral surface of the enterocytes appears to be involved[32]. Such a disruption distorts microvilli and decreases TEER. Our experiments with filter-grown cells, which clearly show dose-related changes in TEER (Fig 5.1), suggest loss of mucosal integrity. These changes are most pronounced after exposure of cells to 10^7 and 10^8 bacteria. On the basis of these findings, it is reasonable to suggest that actin filaments are recruited and remodelled by *S. enteritidis* 857 so as to enter the Caco-2 cells. Like the lectin-induced depolymerization of the actin filaments in differentiated Caco-2 cells[19], the *Salmonella*-induced cytoskeletal changes in Madin-Darby canine kidney cells occur within minutes of infection[18,29].

The potential of enterocyte-like Caco-2 cells to induce the synthesis of Hsp70 and Hsp90 has been investigated in cultures that were exposed to temperatures ranging between 40^0C and 42^0C. In Fig 5.2A and 5.2B it is clearly demonstrated that the levels of the Hsps in differentiated Caco-2 cells increase with increasing temperatures. Also, 5-day-old, undifferentiated cells showed a distinct

heat shock response at 42^0C. Whereas the levels of Hsp70 and Hsp90 in 5-day-old cells were very similar, the response in 19-day-old ones differed considerably.

The results of our investigations also show that after exposure of both crypt-like, undifferentiated and villus-like, differentiated Caco-2 cells to *S. enteritidis* 857, a significant increase in the levels of Hsp70 and Hsp90 could be detected by Western blot analysis (Fig 5.3A, 5.3B, 5.4A, and 5.4B). These Hsps were not of bacterial origin, considering that the monoclonal antibodies did not react with *S. enteritidis* 857 Hsps in either control (37^0C) or heat-shocked (42^0C) bacteria (data not shown). Hsp synthesis has also been induced in Caco-2 cells after incubation with *E. coli C25* or its endotoxin[33]. We were able to confirm the induction of Hsp 70 and Hsp90 by *E. coli* 0111:B4 endotoxin (Fig 5.5A and 5.5B). However, our results clearly showed that *S. enteritidis* 857 endotoxin did not induce the expression of Hsp70 and Hsp90. In addition, neither killed bacteria nor bacteria in the presence of cholamphenicol were able to induce Hsp70 and Hsp90 syntheses (Fig 5.5A and 5.5B). This observation alludes to the fact that another aspect of this bacterium is responsible for these changes. It is tempting to suggest that the invasion process itself is involved. The observed stress response by enterocyte-like Caco-2 cells may be part of a mechanism of protection developed by intestinal epithelial cells in general to deal with potential pathogens in the intestine. The constitutive levels of Hsps in the cells (Fig 5.2A, 5.2B, 5.3A, 5.3B, 5.4A, and 5.4B) are obviously insufficient to protect these cells from invasion by bacteria. Even the increase in Hsps induced by bacterial exposure did not protect the cells. However, such protection may be developed too late. To withstand tissue damage by bacteria and to immediately cope with this damage, cells would most likely benefit from previously induced high levels of Hsps.

Bacteria are known to interfere with the cytoskeleton of gut cells. After the onset of bacterial exposure, depolymerization of the actin filaments of the cytoskeleton takes place almost instantly[18,29]. There is an increasing amount of evidence that emphasizes the relationship between Hsps and the cytoskeleton[34-38]. Induction of Chinese hamster Hsp27 gene expression in mouse NIH/3T3 cells prevents actin depolymerization during acute exposure to cytochalasin[35]. In human colonic epithelial Caco-2/bbe cells, Hsp72 protects the integrity of the actin cytoskeleton against oxidant-induced injury[37]. Liang and MacRae (1997) concluded in their review that Hsp60, Hsp70, Hsp90, and Hsp100 have different but cooperative roles in the formation and function of the eukaryotic cell cytoskeleton. Studies on the function of Hsp70 and Hsp90 revealed the actin-binding activity of these proteins[36], which stabilizes the actin filaments by cross-linking[34]. With respect to Hsp90, this protein was found to bind to at most 10 actin molecules in the polymerized form[39], and its localization in membrane ruffles was revealed by immunofluorescence staining using specific antisera[34]. On the basis of

these findings and our own results (Fig 5.3A, 5.3B, 5.4A and 5.4B), we suggest that high levels of Hsp70 and Hsp90 in Caco-2 cells, which are known to decrease the cellular sensitivity to stress, may be able to inhibit bacteria from adhering and invading through stabilization of the Caco-2 cytoskeleton. If previously induced high levels of Hsps are directed to the stabilization of the cytoskeleton, then bacteria might be unable to use the actin filaments for their own purposes. Further support for this working hypothesis must come from bacterial adherence and invasion experiments with heat-shocked, enterocyte-like Caco-2 cells.

Acknowledgements

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References

1. Lindquist S. 1986. The heat shock response. *Ann Rev Biochem* 55: 1151-1191.
2. Welch WJ. 1992. Mammalian stress response: Cell physiology, structure/function of stress proteins, and implications for medicine and disease. *Physiol Rev* 72: 1063-1081.
3. Craig EA, Gambill BD, Nelson RJ. 1993. Heat shock proteins: molecular chaperones of protein biogenesis. *Microbiol Rev* 57: 402-414.
4. Prohászka Z, Singh M, Nagy K, Kiss E, Lakos G, Duba J, Füst G. 2002. Heat shock protein 70 is a potent activator of the human complement system. *Cell Stress Chaperones* 7: 17–22.
5. Lewis M, Helmsing PJ, Ashburner M. 1975. Parallel changes in puffing activity and patterns of protein synthesis in salivary glands of Drosophila. *Proc Natl Acad Sci USA* 72: 3604-3608.
6. Koninkx JFJG. 1976. Protein synthesis in salivary glands of Drosophila hydei after experimental gene induction. *Biochem J* 158: 623-628.
7. Ovelgönne H, Bitorina M, van Wijk R. 1995. Stressor specific activation of heat shock genes in H35 rat hepatoma cells. *Toxicol Appl Pharmacol* 135: 100-109.
8. Ritossa FM. 1962. A new puffing pattern induced by temperature shock and DNP in Drosophila. Experientia 18: 571-573.
9. Morimoto RI. 1993. Cells in stress: Transcriptional regulation of heat shock genes. *Science* 259: 1409-1410.
10. DiDomenico BJ, Bugaisky GE, Lindquist S. 1982. Heat shock and recovery are mediated by different translational mechanisms. *Proc Natl Acad Sci USA* 79: 6181-6185.
11. Joshi-Barve S, De Benedetti A, Rhoads RE. 1992. Preferential translation of heat shock mRNAs in HeLa cells deficient in protein synthesis initiation factors eIF-4E and eIF-4g. *J Biol Chem* 267: 21038-21043.

12. Li GC, Lazslo A. 1985. Thermotolerance in mammalian cells: a possible role for HSPs,. In: *Changes in Eukaryotic Gene Expression in Response to Environmental Stress,* ed Atkinson BG, Walden DB. New York, Academic Press, 227-254.
13. Landry J, Chrétien P, Lambert H, Hickey E, Weber LA. 1989. Heat shock resistance conferred by expression of the human HSP27 gene in rodent cells. *J Cell Biol* 109: 7-15.
14. Parsell D, Lindquist S. 1994. Heat shock proteins and stress tolerance. In *The Biology of Heat Shock Proteins and Molecular Chaperones,* ed Morimoto RI, Tissières A, Georgopoulos C. Cold Spring Harbour Laboratory Press, NY, 457-494.
15. Hightower LE. 1991. Heat shock, stress proteins, chaperones and proteotoxicity. *Cell* 66: 191-197.
16. Wynn RM, Davie JR, Cox RP, Chuang DT. 1994. Molecular chaperones: Heat-shock proteins, foldases, and matchmakers. *J Lab Clin Med* 124: 31-36.
17. Hendriks HGCJM, Kik MJL, Koninkx JFJG, van den Ingh TS, Mouwen JM. 1991. Binding of kidney bean (*Phaseolus vulgaris*) isolectins to differentiated human colon carcinoma Caco-2 cells and their effect on cellular metabolism. *Gut* 32: 196-201.
18. Koninkx JFJG. 1995. Enterocyte-like Caco-2 cells as a tool to study lectin interaction. In: *Lectins: Biomedical Perspectives,* ed Pusztai A, Bardocz S., Taylor and Francis, London, 81-101.
19. Ovelgönne JH, Koninkx JFJG, Pusztai A, Bardocz S, Kok W, Ewen SW, Hendriks HG, van Dijk JE. 2000. Decreased levels of heat shock proteins in gut epithelial cells after exposure to plant lectins. *Gut* 46: 679-687.
20. Pinto M, Robine-Leon S, Appay MD, Kedinger M, Triadou N, Dussaulx E, Lacroix B, Simon-Assmann P, Haffan K, Fogh J, Zweibaum A. 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma Caco-2 cell line in culture. *Biol Cell* 47: 323-330.
21. Falkow S. 1991. Bacterial entry into eukaryotic cells. *Cell* 65: 1099-1102.
22. Finlay BB, Falkow S. 1997. Common themes in microbial pathogenicity revisited *Microbiol Mol Biol Rev* 61: 136-169.
23. Finlay BB, Falkow S. 1990. *Salmonella* interacts with polarized human intestinal Caco-2 epithelial cells. *J Infect Dis* 162: 1096-1106.
24. van Asten FJAM, Hendriks HGCJM, Koninkx JFJG, van der Zeijst BA, Gaastra W. 2000. Inactivation of the flagellin gene of *Salmonella enterica* serotype *enteritidis* strongly reduces invasion into differentiated Caco-2 cells. *FEMS Microbiol Letters* 185: 175-179.
25. Jepson MA, Colares-Buzato CB, Clark MA, Hirst BH, Simmons NL. 1995. Rapid disruption of epithelial barrier function by *Salmonella typhimurium* is associated with structural modification of intercellular junctions. *Infect Immun* 63: 356-359.
26. Jepson MA, Lang TF, Reed KA, Simmons NL. 1996. Evidence for a rapid, direct effect on epithelial monolayer integrity and transepithelial transport in response to *Salmonella* invasion. *Eur J Physiol* 432: 225-233.
27. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic acid. *Anal Biochem* 159: 76-85.
28. Sanger JM, Chang R, Ashton F, Kaper JB, Sanger JW. 1996. Novel form of actin-based motility transports bacteria on the surfaces of infected cells. *Cell Motil Cytoskeleton* 34: 279-287.
29. Francis CL, Starnbach MN, Falkow S. 1992. Morphological and cytoskeletal changes in epithelial cells occur immediately upon interaction with *Salmonella typhimurium* grown under low-oxygen conditions. *Mol Microbiol* 6: 3077-3087.
30. Galan JE. 1994. *Salmonella* entry into mammalian cells: different yet converging signal transduction pathways? *Trends Cell Biol* 4: 196-199.

31. Francis CL. Ryan TA, Jones BD, Smith SJ, Falkow S. 1993. Ruffles induced by *Salmonella* and other stimuli direct macropinocytosis of bacteria. *Nature* 364: 639-642.
32. Wells CL, van de Westerlo EM, Jechorek RP, Haines HM, Erlandsen SL. 1998. Cytochalasin-induced actin disruption of polarized enterocytes can augment internalization of bacteria. *Infect Immun* 66: 2410-2419.
33. Deitch EA, Beck SC, Cruz NC, De Maio A. 1995. Induction of heat shock gene expression in colonic epithelial cells after incubation with *Escherichia coli* or endotoxin. *Crit Care Med* 23: 1371-1376.
34. Koyasu S, Nishida E, Kadowaki T, Matsuzaki F, Iida K, Harada F, Kasuga M, Sakai H, Yahara I. 1986. Two mammalian heat shock proteins, HSP90 and HSP100, are actin binding proteins. *Proc Natl Acad Sci USA* 83: 8054-8058.
35. Lavoie JN, Gingras-Breton G, Tanguy RM, Landry J. 1993. Induction of Chinese hamster HSP27 gene expression in mouse cells confers resistance to heat shock. HSP27 stabilization of the microfilament organization. *J Biol Chem* 268: 3420-3429.
36. Liang P, MacRae TH. 1997. Molecular chaperones and the cytoskeleton. *J Cell Sci* 110: 1431-1440.
37. Musch MW, Sugi K, Straus D, Chang EB. 1999. Heat shock protein 72 protects against oxidant-induced injury of barrier function of human colonic epithelial Caco-2/bbe cells. *Gastroenterology* 117: 115-122.
38. Xu W, Mimnaugh EG, Kim JS, Trepel JB, Neckers LM. 2002. Hsp90, not Grp94, regulates the intracellular trafficking and stability of nascent ErbB2. *Cell Stress Chaperones* 7: 91–96.
39. Nishida E. Koyasu S, Sakai H, Yahara I. 1986. Calmodulin-regulated binding of the 90 kDa heat shock protein to actin filaments. *J Biol Chem* 261: 16033-16036.

6

MICROBIAL PRODUCTS FROM LACTOBACILLUS CASEI SHIROTA AND L. PLANTARUM 299V INHIBIT SALMONELLA-INDUCED INTERLEUKIN-8 SYNTHESIS AND INDUCE EXPRESSION OF HEAT SHOCK PROTEIN 70 IN ENTEROCYTE-LIKE CACO-2 CELLS

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Abstract

Oral administration of *Lactobacillus* spp as probiotics is gaining importance in the treatment of intestinal inflammations. However, their mechanism of action is still unknown. We investigated the effect of non-specific binding *L. casei* Shirota and mannose-specific *L. plantarum* 299v and their spent culture supernatants (SCS) on *Salmonella enteritidis* 857 growth, interleukin (IL)-8 and heat shock protein (Hsp) 70 synthesis in a human intestinal cell line. Crypt- and villus-like Caco-2 cells were infected by *S. enteritidis* 857 (1-200 bacteria/cell) after or without prior exposure to the lactobacilli (1-500 bacteria/cell, 30 min) or by co-incubation of *S. enteritidis* 857 and the lactobacilli (1 h). In another experiment, cells were exposed for 1 h to SCS or *S. enteritidis* 857 (100 bacteria/cell) pretreated in the SCS (1 h). The effect of the lactobacilli SCS on *S. enteritidis* 857 growth was evaluated by the agar plate diffusion test. IL-8 and Hsp70 were assessed over 2-24 h using ELISA and Western blotting, respectively. Neither *L. casei* Shirota nor *L. plantarum* 299v affected the *S. enteritidis* 857 growth and IL-8 production. In addition, they did not induce Hsp70 expression by Caco-2 cells. Instead, their SCS inhibited the *S. enteritidis* 857 growth and IL-8 production and induced the expression of Hsp70 by both crypt- and villus-like cells. We conclude that the beneficial effect of *Lactobacillus* spp to the intestinal inflammations might be associated with a decrease of the IL-8 levels. Our results clearly show that this effect could be mediated, at least in part, via a secreted antimicrobial product(s) either directly against the pathogens or indirectly through the synthesis of Hsp70.

Introduction

The intestinal epithelium plays a crucial role in the pathogenesis of infections caused by a variety of pathogenic microbes as well as in the host defence against them. During its colonization by enteropathogenic bacteria like *Salmonella enteritidis* 857, the intestinal epithelium serves as the first line of defence against the invading microbes and reacts to the colonization with an instant innate immune response. This localized response involves the rapid expression and up-regulation of several proinflammatory cytokines, predominantly interleukin (IL)-8. This interleukin attracts and directs neutrophils to the site of inflammation, a response that is vital to the inflammatory diarrhoea caused by *Salmonella* infection[1-3]. In addition, the intestinal epithelial cells produce putative protective proteins, the heat shock proteins (Hsps), in response to various stresses such as heat, infection and inflammation. These Hsps confer cytoprotection against the stresses by acting as chaperones through protein-protein interaction[4,5]. Their protection against intestinal inflammation involves inhibition of persistent secretion of inflammatory cytokines such as IL-8[6]. Because of their ability to suppress the IL-8 production, Hsps might be useful in the treatment of *S. enteritidis* 857 inflammations.

Lactic acid bacteria such as lactobacilli and bifidobacteria, are normal inhabitants of the human gastrointestinal tract that constitute a considerable proportion of the gut flora. Selected strains from these genera exert a positive influence on host health or physiology when ingested[7]. After oral administration, they survive the gastrointestinal passage, improve the intestinal microbial balance and confer protection against potential enteropathogenic bacteria[8,9]. Their protective effects are mediated via several mechanisms like production of various acids, hydrogen peroxide or bacteriocins, competition for nutrients or adhesion receptors, anti-toxin actions, stimulation of the immune system and suppression of cytokine production by intestinal epithelial cells including IL-8, transforming growth factor (TGF)-β, and tumour necrosis factor (TNF)-α[10,11]. In addition to their beneficial effects in the gastrointestinal tract, lactobacilli have been shown to protect other systems. In the rat for instance, intravenously administered lactobacilli cultivation products reduce reperfusion tachyarrhythmia and improves functional recovery of the ischemized heart. These beneficial effects are associated with overexpression of the protective protein, the Hsp70[12].

Several studies have implicated the use of *Lactobacillus casei* Shirota and *L. plantarum* 299v to improve specific local intestinal inflammations such as ulcerative and *Clostridium difficile* colitis, Crohn's disease, pouchitis, and diarrheagenic illnesses[13-15]. Most, if not all, of these disorders are characterized by high levels of IL-8 production[16,17]. Patients significantly benefit from interventions that decrease these levels[18-21]. It is not well established whether the mechanism

underlying the benefits of *L. casei* Shirota and *L. plantarum* 299v in the improvement of the intestinal inflammations is linked to the regulation of IL-8. However, the observations that certain strains of *Lactobacillus* spp are capable of suppressing the IL-8 production by the intestinal epithelial cells[11] and inducing expression of Hsp70 in the heart[12], suggest that the relative importance of *L. casei* Shirota and *L. plantarum* 299v in the intestinal inflammations might involve the modulation of the chemokine IL-8, either direct or indirect through an overexpression of Hsp70. Specific to *Salmonella* infections, *L. casei* Shirota and *L. plantarum* 299v might differ in their protective role due to receptor competition. The pathogenic effects of *Salmonella* follow the bacterial stereo-specific adhesin-receptor interaction that involves mannose moieties on the human intestinal surface[22]. Adhesion of the bacterium to these mannose receptors is essential for its subsequent pathogenicity and agents competing for similar receptors might alter the *S. enteritidis* effects. Since adhesion of probiotics to the intestinal surface is considered a prerequisite for the competitive exclusion of pathogens and for the modulation of local and systemic immunological activities[23,24], *Lactobacillus* spp that vary in their competition with *S. enteritidis* to enterocyte receptors may have different effects on the pathogen. *L. casei* Shirota (non-specific binding) and *L. plantarum* 299v (mannose-specific) could therefore, be suitable representative models for *Lactobacillus-Salmonella* interaction with the intestinal epithelium.

To gain more insight into the mechanisms involved in the beneficial effect of *Lactobacillus* spp on enteropathogenic *S. enteritidis* 857, a cause of IL-8 production and subsequent life-threatening diarrheic gastroenteritis in human[1-3], we examined the effect of *L. casei* Shirota and *L. plantarum* 299v on *S. enteritidis* 857-induced IL-8 production by intestinal epithelial cells. Specifically, we explored the effect of *L. casei* Shirota and *L. plantarum* 299v and their cultivation products or spent culture supernatants (SCS) on *S. enteritidis* 857 growth and IL-8 production. We also assessed their ability to induce Hsp70 that is known to inhibit IL-8 production by intestinal epithelial cells. The study was conducted in enterocyte-like Caco-2 cells, an in vitro model of the human intestinal epithelium that differentiate in culture and acquire characteristics both structurally and functionally of either crypt cells (5-day-old Caco-2 cells) or villus cells (19-day-old Caco-2 cells) of the small intestine[25,26].

Materials and methods

Cell culture

Human colon adenocarcinoma Caco-2 cells (ATCC HTB 37) were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 1% (v/v) non-

essential amino acids, 10 mM $NaHCO_3$, 1.7 mM glutamine, 50 µg ml^{-1} gentamicin, 25 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethane-sulfonic acid (4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid) (all Flow Laboratories, Amstelstad B.V., Amsterdam, The Netherlands), and 20% (v/v) fetal calf serum (Sanbio B.V., Uden, The Netherlands). Supplemented culture medium devoid of gentamicin and fetal calf serum in the experiments is referred to as plain DMEM. Cell cultures were maintained at 37°C in 95% air-5% CO_2 in a humidified atmosphere with three cell culture medium changes per week. Cells were seeded at 40 000 cells cm^{-2} in 25 cm^2 tissue culture flasks (Greiner, Alphen a/d Rijn, The Netherlands) containing 5 ml cell culture medium. This study encompassed 25 cell passages of the cell line ranging from the 5^{th} to the 29^{th}.

Bacterial strains

DeMan, Rogosa, Sharpe (MRS) broth (Oxoid LTD., Basingstoke, Hampshire, England) (100ml) was inoculated with 1 ml of a stationary culture of *L. casei* Shirota or *L. plantarum* 299v and grown for 24 h at 37°C. To obtain bacteria, the bacterial culture was centrifuged for 15 min at 1500*g* (20°C), whereas to obtain spent culture supernatants (SCS), the bacterial culture was centrifuged differently (30 min 10 000*g* at 4°C). The collected bacteria were subsequently suspended in plain DMEM and serial dilutions were made to obtain different number of bacteria. The SCS were used to treat *S. enteritidis* 857 before exposure of the bacteria to the cells and in agar plate diffusion tests.

One colony of *S. enteritidis* 857 grown on Luria-Bertani (LB) agar was inoculated into 5 ml LB broth. The inoculum was then grown overnight (16 h) with shaking (200 rpm) at 37°C. The resulting bacterial suspension was inoculated into LB broth at 1:100 (v/v) followed by incubation with shaking (200 rpm) at 37°C for 2 h to obtain logarithmically growing bacteria. After this growth, the bacteria were collected by centrifugation (15 min 1500*g* at 20°C) and finally suspended in plain DMEM after or without various pretreatments.

Preparation of SCS and pretreatment of S. enteritidis 857

Collected SCS from *L. casei* Shirota and *L. plantarum* 299v were filtered through a sterile 0.22-µm-pore-size filter unit (Schleider & Schuell GmbH, Dassel, Germany) and concentrated two-fold by freeze-drying. Since the pH of the MRS broth of a 24-h culture of lactobacilli appeared to be 4.5, two MRS controls have been used: an MRS control adjusted to pH 4.5 with dilute lactic acid (MRS-lactic acid) (final concentration 100 mM) and an MRS control adjusted to pH 4.5 with

hydrochloric acid (MRS-HCl). *S. enteritidis* 857 was pretreated for 1 h at 37°C by incubating in 25 ml SCS-LcS, SCS-Lp, MRS-HCl, MRS-lactic acid, plain DMEM-HCl (all pH 4.5) or plain DMEM (pH 7.4) and then collected by centrifugation (15 min 1500*g* at 37°C). The bacteria were washed once with 10 ml 0.01 M phosphate-buffered saline (PBS) (0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, 0.9% (w/v) NaCl), pH 7.3 and finally re-suspended in plain DMEM (pH 7.4) before infecting the cells.

Infection of Caco-2 cells by L. casei Shirota, L. plantarum 299v and S. enteritidis 857

In one set of experiments, Caco-2 cells were infected with *L. casei* Shirota, *L. plantarum* 299v or *S. enteritidis* 857 (0-200 bacteria/cell) for 1 h. In the second set of experiments, cells were infected with a combination of either of the lactobacilli (LB) (500 LB/cell) and *S. enteritidis* 857 (Se) (0-200 Se/cell). The exposure in the latter set was either coincubation of one *Lactobacillus* and *S. enteritidis* 857 (1 h) or pre-incubation of one *Lactobacillus* (30 min) followed by *S. enteritidis* 857 (1 h) without removal of the *Lactobacillus*. In the third set of experiments, Caco-2 cells were infected with *S. enteritidis* 857 (100 Se/cell, 1 h) pretreated for 1 h in 25 ml SCS-LcS, SCS-Lp, MRS-HCl, MRS-lactic acid, plain DMEM-HCl (all pH 4.5) or plain DMEM (pH 7.4). The exposure to bacteria in all experiments was stopped by washing the cells twice with 5 ml plain DMEM supplemented with 50 µg ml^{-1} gentamicin. Cells were then allowed to recover for 2, 4, 6, 8, 16, or 24 h at 37°C in a humidified atmosphere of 95% air-5% CO_2.

Collection of the cell culture medium and the Caco-2 cells

The cell culture medium was collected and centrifuged (10 min 600*g* at 4°C) to obtain the supernatants. After collecting the cell culture medium, the cell monolayers were rinsed twice with 5 ml PBS (37°C). The monolayers were then incubated for 30 min at 37°C with 5 ml transfer medium, pH 7.3 (8 g l^{-1} NaCl, 0.2 g l^{-1} KH_2PO_4, 0.2 g l^{-1} $Na_2EDTA.2H_2O$). Subsequently, the dispersed cells were collected in tubes and washed twice with 10 ml PBS (0°C). The washing procedure involved centrifugation (10 min 300*g* at 4°C) and discarding of supernatants. After the last washing step, the cells were collected by centrifugation (10 min 600*g* at 4°C). The collected cell culture supernatants and the cells were then stored at -70°C until being analyzed.

Effect of L. casei Shirota and L. plantarum 299v SCS on S. enteritidis 857 growth

Agar plate diffusion test was used to determine the effect of SCS from *L. casei* Shirota and *L. plantarum* 299v, MRS, plain DMEM, and exogenous human Hsp70 on the growth of *S. enteritidis* 857. Sterilized (121^0C, 15 min) nutrient agar [1g l^{-1} lab lemco broth, 2 g l^{-1} yeast extract, 5 g l^{-1} pepton water, 15 g l^{-1} agar bacteriological (all Oxoid), 5 g l^{-1} NaCl] (pH 7.4) was dispensed in petri dishes. A 14-mm-diameter gel punch was used to make 1 or 2 wells per dish. A total volume of 450 µl (3x150 µl) from each sample was added to the respective well. After addition of every 150 µl, the dishes were incubated (50^0C) to speed up the diffusion. From the 16-h-grown *S. enteritidis* 857, 500 µl of $1x10^4$ or 10^5 CFU ml^{-1} was added to 5 ml MRS broth (45^0C) containing 0.7% agar. After a rapid dispersal with a laboratory vortex mixer, the agar was poured into the dishes. The dishes were then incubated overnight at 37^0C followed by assessment of the diameters of the inhibition zones.

Western blot analysis

Distilled water (4^0C) was added to the collected Caco-2 cells (0.4 ml for crypt- and 1.0 ml for villus-like) and the mixture was sonicated (0^0C, 30 sec at an amplitude of 24 µm amplitude) with an MSE Soniprep 150 (Beun de Ronde BV, Abcoude, The Netherlands). The protein content of the resulting sonicates was determined[27] and found to be equal in all samples. One-third volume of loading buffer (3x the strength) (125 mM Tris-(hydroxymethyl)aminomethane-HCl, 4% sodium dodecyl sulfate (SDS), 10% ß-mercaptoethanol, 20% glycerol, and 0.0015% bromophenolblue, pH 6.8) was added to the protein samples and the mixture was heated (95^0C, 5 min). Equal amounts of proteins (10 µg) were loaded on the slots of the gel and were separated using 10% SDS polyacrylamide gelelectrophoresis gels. A protein ladder (Bio-Rad Laboratories, California, USA) was loaded as a molecular weight marker. The proteins were then transferred to Immobilon-P polyvinylidene difluoride membrane following the recommendations of the manufacturer (Millipore, Bedford, USA). Staining with Ponceau Red stain confirmed the protein transfer. The non-specific binding sites on the membrane were blocked by incubating the membrane for 1 h with 10 ml of blocking solution (0.1% Tween/PBS, pH 7.3 containing 5% Boehringer blocking agent) (Amersham Pharmacia Biotech, Buckinghamshire, UK) and the membrane was washed with 50 ml of washing buffer (0.1% Tween/PBS, pH 7.3). The washing procedure consisted of two quick washes followed by three additional ones (1x15 min, 2x5 min). Subsequently, the membrane was incubated for 1 h with 2.5 µg of mouse

anti-Hsp70 monoclonal antibody (SPA-810) (Stressgen Biotechnologies Corporation, Victoria, British Columbia, Canada) in 5 ml blocking solution (0.1% Tween/PBS, pH 7.3) containing 0.5% Boehringer blocking agent. After repeating the above washing procedure, the blots were incubated for 1 h with 5 µg of goat anti-mouse IgG alkaline phosphatase secondary antibody (SAB-101) (Stressgen) in 10 ml blocking solution (0.1% Tween/PBS, pH 7.3) containing 0.5% Boehringer blocking agent. The washing procedure was repeated again and the blots were incubated for 5 min in 10 ml alkaline phosphatase detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 5 mM $MgCl_2.6H_2O$, pH 9.5) to which 35 µl nitroblue tetrazolium chloride (100 mg ml^{-1}) (product number 1383213) and 35 µl 5-bromo-4-chloro-3-indoyl-phosphate (50 mg ml^{-1}) (product number 1383221) (both from Roche Diagnostics GmbH, Mannheim, Germany) were added.

Determination of IL-8 secretion by sandwich ELISA

IL-8 concentrations were assayed using the IL-8 Cytosets™ antibody pair kit containing matched, pre-titered and fully optimized capture and detection antibodies, recombinant standard and streptavidin-horseradish peroxidase (catalog number CHC1304) (Biosource Europe S.A., Nivelles, Belgium). The assay was done according to the manufacturer's specifications.

Statistical analysis

Statistical significance between the mean values of control, *L. casei* Shirota, *L. plantarum* 299v, lactobacilli SCS and *S. enteritidis* 857 exposed cells was assessed by one-way analysis of variance (ANOVA) with comparison of means. Differences were considered significant at 95% confidence interval using the Student's T-test.

Results

Induction of IL-8 expression by L. casei Shirota, L. plantarum 299v, and S. enteritidis 857 in human enterocyte-like Caco-2 cells

Before investigating the effect of *L. casei* Shirota and *L. plantarum* 299v on the *S. enteritidis* 857-induced expression of IL-8 by enterocyte-like Caco-2 cells, a chemoattractant that is pivotal to most intestinal inflammations, the pattern of IL-8 secretion induced by each strain was determined.

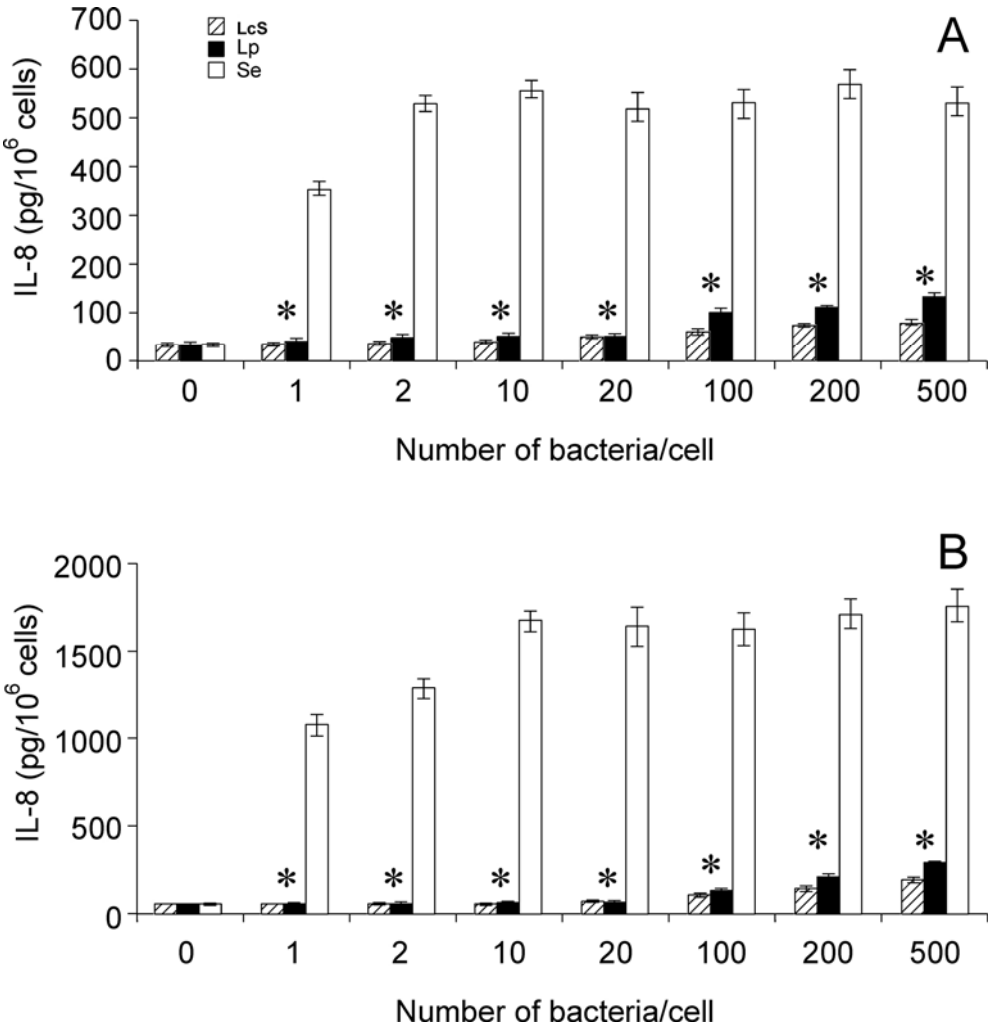

Fig 6.1. Dose dependent induction of IL-8 production by Lactobacillus casei Shirota, L. plantarum 299v, and Salmonella enteritidis 857 in human enterocyte-like Caco-2 cells after exposure. *Crypt- (A) or villus- (B) like Caco-2 cells were exposed to 0, 1, 2, 10, 20, 100, 200, or 500 bacteria/cell for 1 h in plain Dulbecco modified Eagle medium (DMEM). After 24 h of recovery in plain DMEM containing gentamicin, the cell culture medium was collected and IL-8 levels were measured using ELISA. The results are expressed as pg IL-8/10^6 cells. The IL-8 levels were determined using two cell passages and triplicate cultures per passage. Significant differences (*$p<0.05$) between the IL-8 levels of S. enteritidis 857-exposed cells and L. casei Shirota or L. plantarum 299v cells are indicated. (LcS: L. casei Shirota; Lp: L. plantarum; Se: S. enteritidis 857).*

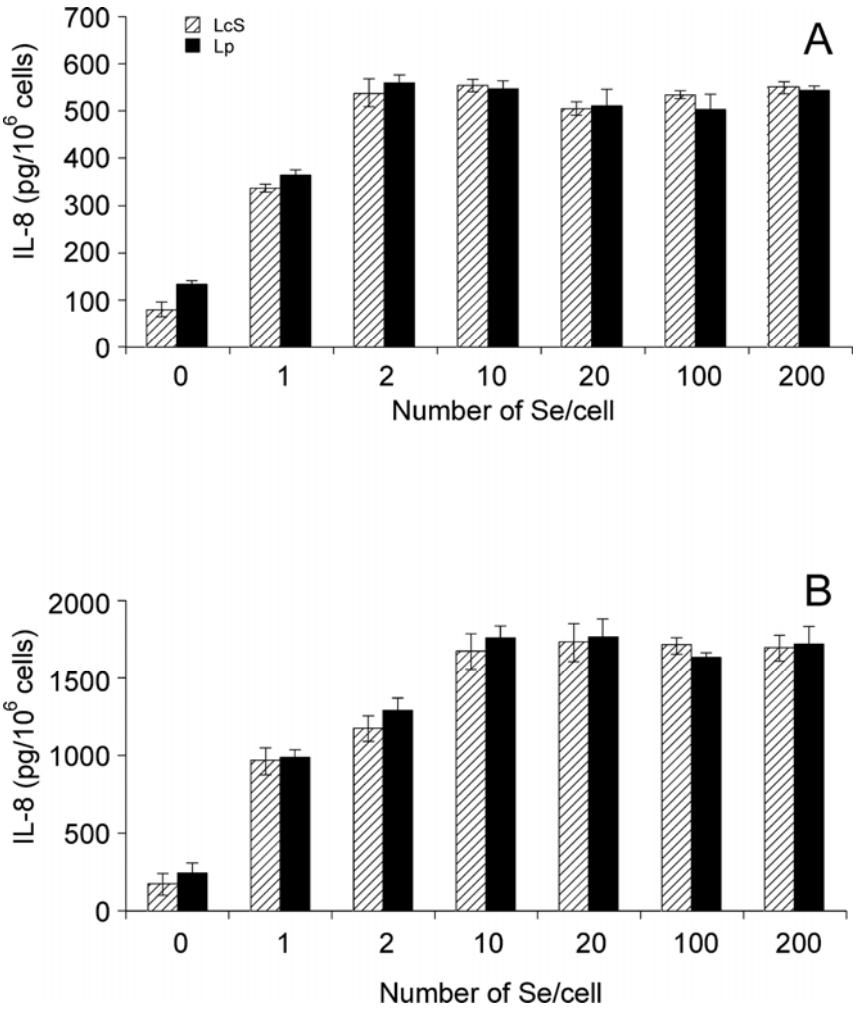

Fig 6.2. Dose dependent induction of IL-8 production by Salmonella enteritidis 857 in Caco-2 cells after pre-incubation with Lactobacillus casei Shirota or L. plantarum 299v. *Crypt- (A) or villus- (B) like Caco-2 cells were pre-incubated for 30 min to L. casei Shirota or L. plantarum 299v (500 bacteria/cell) and subsequently exposed to S. enteritidis 857 (0, 1, 2, 10, 20, 100, 200, or 500 bacteria/cell) for 1 h without removal of the lactobacilli. The exposure was done in plain Dulbecco modified Eagle medium (DMEM). After 24 h of recovery in plain DMEM containing gentamicin, the cell culture medium was collected and IL-8 levels were measured using ELISA. The results are expressed as pg IL-8/10^6 cells. The IL-8 levels were determined using two cell passages and triplicate cultures per passage. There were no significant differences ($p<0.05$) between the IL-8 levels in either combination. ((LcS and Lp, are levels of IL-8 in Caco-2 cells exposed to S. enteritidis 857 (Se) after pre-incubation with L. casei Shirota and L. plantarum 299v, respectively)).*

Exposure of the cells to graded numbers of bacteria (1, 2, 10, 20, 100, 200, and 500 bacteria/cell) for 1 h, resulted in a linear dose-dependent production of IL-8 (Fig 6.1A, 6.1B). A significant increase in the IL-8 levels in comparison with control cells was exhibited differently with the three bacterial strains. Already on exposure to low numbers of *S. enteritidis* 857 (1 Se/cell) the levels of IL-8 secretion rapidly increased and reached the highest values after incubation with 2 or 10 Se/cell for crypt- (Fig 6.1A) or villus- (Fig 6.1B) like cells respectively. When the numbers of *S. enteritidis* 857 were further increased up to 500 Se/cell, the levels of IL-8 remained approximately constant. By contrast, low numbers of *L. casei* Shirota and *L. plantarum* 299v (1-20 LB/cell) had little or no effect at all on IL-8 production by both crypt- (Fig 6.1A) and villus- (Fig 6.1B) like cells. Higher numbers (100-500 LB/cell) of either *Lactobacillus* induced a slight but significant linear dose-dependent production of IL-8 by Caco-2 cells. Compared with the *Salmonella*-induced IL-8 production, the levels of IL-8 induced by the lactobacilli at any equivalent bacterial numbers, were far lower than those induced by *S. enteritidis* 857. Fig 6.1A, 6.1B also show a modest but significant difference between *L. casei* Shirota- and *L. plantarum* 299v-induced IL-8 production with *L. casei* Shirota having lower levels than *L. plantarum* 299v. The difference was observed at 100-500 LB/crypt-like cell and 200-500 LB/villus-like cell.

Effect of L. casei Shirota and L. plantarum 299v on S. enteritidis 857-induced IL-8 synthesis by Caco-2 cells

To establish whether exposure of Caco-2 cells to lactobacilli might affect the pattern of *S. enteritidis* 857-induced IL-8 synthesis, in one set of experiments, we exposed the cells to lactobacilli (500 LB/cell) for 30 min prior to infection with *S. enteritidis* 857 (0-200 Se/cell) for 1 h. The infection with the pathogen was performed in the presence of the lactobacilli. As shown in Fig 6.2A, 6.2B it is clear that *L. casei* Shirota and *L. plantarum* 299v did not affect the pattern of IL-8 synthesis induced by *S. enteritidis* 857 in both crypt- and villus-like Caco-2 cells. This pattern was similar to that observed when Caco-2 cells were exposed to *S. enteritidis* 857 alone (Fig 6.1A, 6.1B). The levels of IL-8 produced in response to either *L. casei* Shirota or *L. plantarum* 299v alone were raised further after combined exposure with *S. enteritidis* 857. In both crypt- (Fig 6.2A) and villus- (Fig 6.2B) like cells, the secreted IL-8 levels rapidly increased on combined exposure with *S. enteritidis* 857 (1 Se/cells) and reached their highest levels at 2 Se/crypt-like cell or 10 Se/villus-like Caco-2 cell. With further increasing numbers of *S. enteritidis* 857 the IL-8 levels remained approximately constant.

In Fig 6.3A, 6.3B the time-dependent changes in the levels of IL-8 in Caco-2 cells co-incubated with *L. casei* Shirota or *L. plantarum* 299v (500 LB/cell)

and *S. enteritidis* 857 (100 Se/cell) for 1 h are presented. Compared with the unexposed Caco-2 cells (Fig 6.1A, 6.1B) exposure to *L. casei* Shirota or *L. plantarum* 299v resulted in a gradual increase of the IL-8 levels that became significant after 16 h of recovery and reached their highest levels after 24 h. At 16 and 24 h after recovery the IL-8 levels induced by *L. casei* Shirota were significantly lower than those induced by *L. plantarum* 299v. Exposure of Caco-2 cells to *S. enteritidis* 857 alone or in co-incubation with either *L. casei* Shirota or *L. plantarum* 299v yielded a common pattern of IL-8 production with little or no effect at all of *L. casei* Shirota or *L. plantarum* 299v on *S. enteritidis* 857-induced IL-8 production in enterocyte-like Caco-2 cells (Fig 6.3A, 6.3B). In all treatments and in both cell types, the IL-8 levels were already significantly higher after 4 h of recovery and kept increasing. The levels peaked at 16 h and remained constant in crypt-like cells (Fig 6.3A), but kept increasing throughout the recovery time in villus-like Caco-2 cells (Fig 6.3B). When compared with the expression levels of IL-8 induced by *L. casei* Shirota or *L. plantarum* 299v at 24 h, exposure to *S. enteritidis* 857 increased these level about five-fold at this time point.

Inhibition of Salmonella-induced IL-8 production by lactobacilli SCS in Caco-2 cells

To investigate the effect of SCS from *L. casei* Shirota or *L. plantarum* 299v on *S. enteritidis* 857-induced synthesis of IL-8 by enterocyte-like Caco-2 cells the cells were exposed for 1 h to pre-treated *S. enteritidis* 857 (100 Se/cell). Pre-treatment consisted of a 1 h incubation period of the pathogen with either SCS-LcS, SCS-Lp, MRS-HCl, MRS-lactic acid, plain DMEM-HCl (all pH 4.5) or plain DMEM (pH 7.4). As indicated in Fig 6.4A, 6.4B a marked decrease in *S. enteritidis* 857-induced IL-8 secretion was observed when the Caco-2 cells were infected with *S. enteritidis* 857 pre-treated with the SCS of lactobacilli. The decrease of the IL-8 levels was observed throughout the recovery period (2-24 h) in both crypt-like and villus-like cells. In crypt-like cells the inhibition was most pronounced after 4 h. At this time point the IL-8 levels were about 7-fold lower than in cells exposed to *S. enteritidis* 857 pre-treated with plain DMEM (untreated pathogen) (Fig 6.4A). The most conspicuous decrease of the IL-8 levels in villus-like cells was observed after 2 h of recovery. Compared to the cells infected with the untreated pathogen the IL-8 levels were 10-fold lower (Fig 6.4B). It was found that *S. enteritidis* 857 treated with MRS-HCl or MRS-lactic acid also lowered the IL-8 levels. However, compared to the SCS-LcS and SCS-Lp treatment the IL-8 decreasing activity was much smaller. No differences in IL-8 decreasing activity between the SCS of *L. casei* Shirota and *L. plantarum* 299v could be observed.

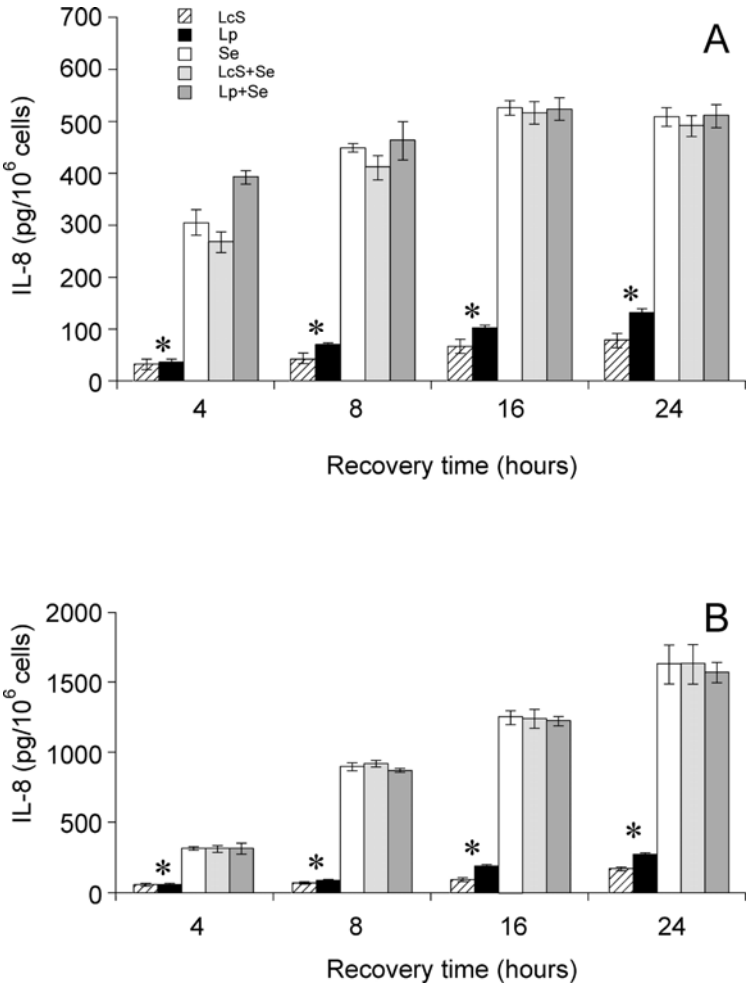

Fig 6.3. Time-course induction of IL-8 production by Lactobacillus casei Shirota and L. plantarum 299v with or without co-incubation with Salmonella enteritidis 857 in Caco-2 cells. *Crypt- (A) and villus- (B) like Caco-2 cells were exposed to L. casei Shirota or L. plantarum 299v (500 bacteria/cell) co-incubated with S. enteritidis 857 (100 bacteria/cell) for 1 h in plain Dulbecco modified Eagle medium (DMEM). After recovery for 4, 8, 16, or 24 h in plain DMEM containing gentamicin, culture medium was collected and IL-8 levels were measured using ELISA. The results are expressed as pg IL-8/10^6 cells. IL-8 secretion was determined using two cell passages and triplicate cultures per passage. Significant differences (*$p<0.05$) between the IL-8 levels of cells exposed to S. enteritidis 857 alone or in co-incubation with L. casei Shirota or L. plantarum 299v and cells exposed to L. casei Shirota or L. plantarum 299v are indicated. (LcS: L. casei Shirota; Lp: L. plantarum 299v; Se: S. enteritidis 857; LcS+Se: co-incubation of L. casei Shirota and S. enteritidis 857; LcS+Lp: co-incubation of L. plantarum 299v and S. Enteritidis 857).*

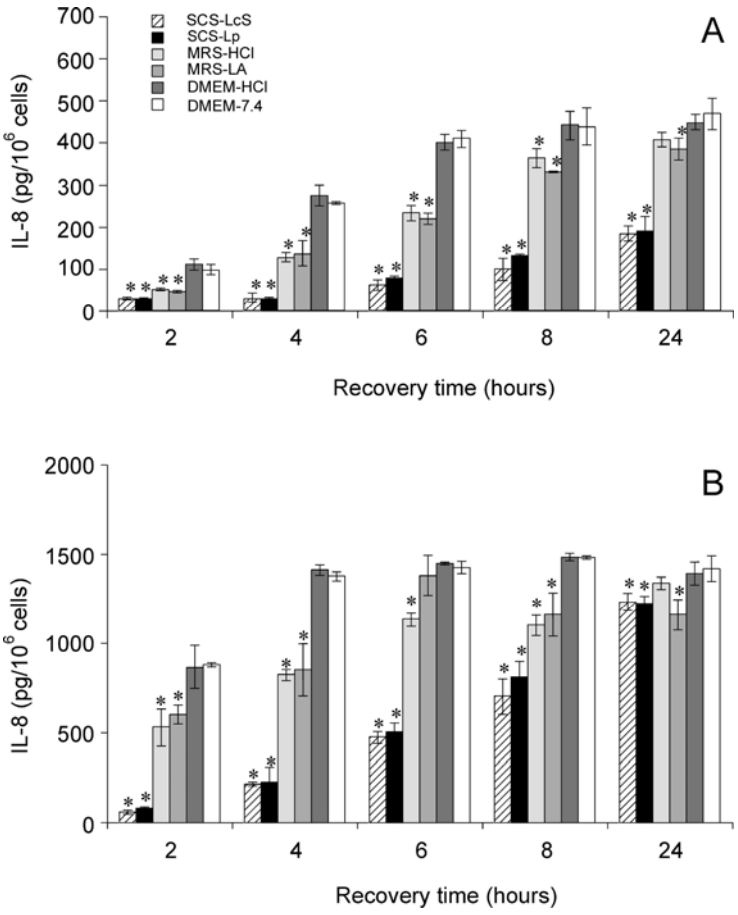

Fig 6.4. Inhibition of Salmonella enteritidis 857-induced IL-8 production by spent culture supernatant (SCS) from Lactobacillus casei Shirota and L. plantarum 299v in Caco-2 cells. *Crypt- (A) and villus- (B) like Caco-2 cells were exposed for 1 h to S. enteritidis 857 (100 bacteria/cell) in plain Dulbecco modified Eagle medium (DMEM). Before exposure, the S. enteritidis 857 were pretreated in either lactobacilli spent culture supernatant (SCS), MRS broth, or plain DMEM for 1 h. After a recovery period of 2, 4, 6, 8, or 24 h in plain DMEM containing gentamicin, the culture medium was collected and IL-8 levels were measured using ELISA. The results are expressed as pg IL-8/10^6 cells. IL-8 secretion was established using 2 cell passages and triplicate cultures per passage. Significant differences (*$p<0.05$) between the IL-8 levels of cells exposed to S. enteritidis 857 pretreated with plain DMEM and those pretreated with spent culture supernatant from L. casei Shirota or L. plantarum 299v and MRS broth are indicated. (SCS-LcS, SCS-Lp: spent culture supernatants from L. casei Shirota or L. plantarum 299v, respectively; MRS-HCl, MRS-LA: MRS broth adjusted to pH 4.5 with hydrochloric acid or lactic acid, respectively; DMEM-HCl, DMEM-7.4: plain DMEM adjusted to pH 4.5 with hydrochloric acid or plain DMEM at pH 7.4, respectively).*

The IL-8 levels of both crypt-like and villus-like cells exposed to SCS-LcS and SCS-Lp pre-treated salmonellae steadily increased in a time dependent manner. Although the IL-8 increase in villus-like cells was much faster, a significant difference remained present after 24 h of recovery between these cells and those infected with the untreated pathogen (Fig 6.4).
Variations in pH (4-7.4) or exposure to lactic acid (1-100 mM) had no effect on IL-8 production by Caco-2 cells (data not shown).

Lactobacilli SCS inhibits the growth of S. enteritidis 857

The results of the agar plate diffusion tests, which are presented in Table 6.1, clearly showed that the SCS from either *L. casei Shirota* or *L. plantarum* 299v inhibited the growth of *S. enteritidis* 857. The widths of the inhibition rings displayed by MRS-broth (2-fold concentrated; pH 4.5) and plain DMEM were significantly smaller than the SCS-induced ones. When compared with these values, a 12-fold wider ring of *S. enteritidis* 857 growth inhibition was achieved with the SCS of *L. casei Shirota* and *L. plantarum* 299v.

Table 6.1 Inhibition of Salmonella enteritidis 857 growth by L. casei Shirota or L. plantarum 299v spent culture supernatants.

Treatment	Width of the inhibition zone (mm)
Plain DMEM	3.0 ± 1.2
MRS-broth	2.8 ± 0.8
SCS-LcS	*17.5 ± 1.2
SCS-Lp	*18.5 ± 1.4

Spent culture supernatants (SCS) from *L. casei* Shirota or *L. plantarum* 299v were loaded in nutrient agar holes followed by pouring of *S. enteritidis* 857 suspension. After overnight incubation at 37^0C, growth inhibition rings were measured around the holes. Two experiments were performed to determine the width of the inhibition rings. Significant differences (*$p<0.05$) between the inhibition zones for holes containing SCS from *L. casei* Shirota or *L. plantarum* 299v and those containing plain Dulbecco modified Eagle medium (DMEM) or MRS-broth are indicated.

Expression of Hsp70 in enterocyte-like Caco-2 cells after exposure to SCS-treated S. enteritidis 857

Because the expression of Hsp70 is associated with the protection of intestinal epithelial cells, we investigated whether the SCS from either *L. casei* Shirota or *L. plantarum* 299v was able to modify the expression of Hsp70 in Caco-

2 cells. When the cells were exposed for 1 h to the SCS-treated salmonellae, increasing levels of Hsp70 could be established in both crypt- and villus-like Caco-2 cells (Fig 6.5). The expression appeared to be time-dependent and reached their highest levels 24 h after recovery from exposure to SCS-treated salmonellae. The highest SCS-induced Hsp70 levels were only slightly lower than those of heat-shocked control cells. There was no induction of Hsp70 expression when cells were incubated with the *L. casei Shirota, L. plantarum* 299v or salmonellae exposed to MRS-HCl, MRS-lactic acid, or plain DMEM at pH 4.5 or 7.4 (data not shown).

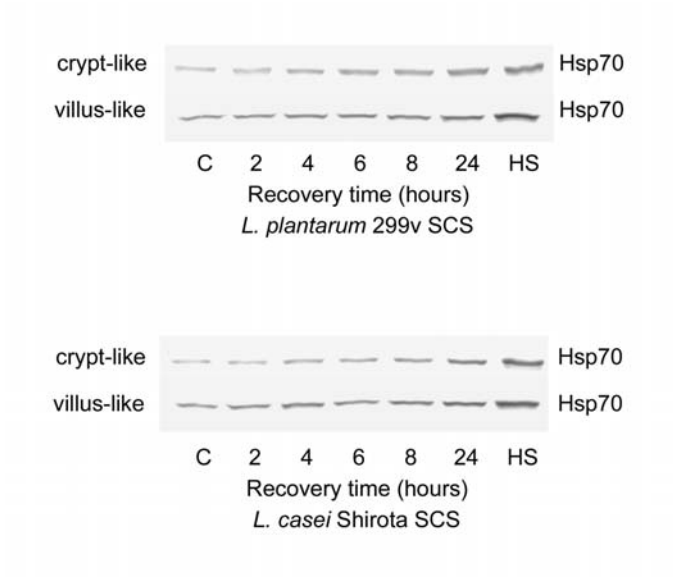

Fig 6.5. Expression of Hsp70 by enterocyte-like Caco-2 cells after exposure to Salmonella enteritidis 857 pretreated in Lactobacillus casei Shirota or L. plantarum 299v spent culture supernatants. *Crypt- or villus-like Caco-2 cells were exposed to Salmonella enteritidis 857 pretreated in spent culture supernatants (SCS) from L. casei Shirota or L. plantarum 299v for 1 h. After 2-24 h of recovery in plain Dulbecco modified Eagle medium (DMEM) containing gentamicin, cells were processed for Western blotting and immunostaining. (C: levels of Hsp70 in control Caco-2 cells; HS: levels of Hsp70 in heat shocked cells (1 h at 42^0C; 6 h recovery at 37^0C)].*

Discussion

Cytoprotective properties of *Lactobacillus* displayed in the intestinal epithelium have been demonstrated in many recent studies. The ability to stimulate

the host's nonspecific immunity, to suppress the inflammatory cytokines (TNF-α, TGF-β, IL-8), to impede the invasion, and to inhibit the promoted cellular injuries and intracellular lifestyle of pathogens, are established probiotic beneficial features[11,28-30]. The present study shows that exposure of enterocyte-like Caco-2 cells to nonspecific receptor binding *L. casei* Shirota and mannose-specific *L. plantarum* 299v induces a modest IL-8 production (Fig 6.1) with no effect on *S. enteritidis* 857-induced IL-8 production (Fig 6.2, 6.3). Instead, their antimicrobial products, which are secreted in the SCS, inhibit the IL-8 production (Fig 6.4) and the growth of *S. enteritidis* 857 (Table 6.1), and induce the expression of Hsp70 (Fig 6.5).

In a normal intestinal environment, the commensal flora conditions the level of activation of the mucosal immune response and plays a vital role in driving the mucosal inflammation in genetically susceptible individuals. This activation involves the expression and secretion of various inflammatory cytokines, including the chemoattractant IL-8[17], at constitutive levels that are considered insignificant to alter the normal microenvironment[30]. The levels of IL-8 induced by either *L. casei* Shirota or *L. plantarum* 299v observed in this study, were very low when compared with those induced by the pathogenic *S. enteritidis* 857 (Fig 6.1). Indeed, the highest levels induced after 24 h were just slightly higher than those of control cells, alluding that they might be within the range of constitutive expression responsible for normal immune surveillance *in vivo*.

Our results in this study clearly show that both *L. casei* Shirota and *L. plantarum* 299v are incapable of modulating the *Salmonella*-induced IL-8 production in Caco-2 cells (Fig 6.2, 6.3). In any case, when Caco-2 cells were treated with *S. enteritidis* 857 in the presence of either *Lactobacillus*, the patterns of IL-8 observed were similar to that induced by *S. enteritidis* 857 alone. It is interesting to note that even exposure to 500 LB/cell could not affect the IL-8 production by Caco-2 cells exposed to the lowest number of *S. enteritidis* 857 (1 Se/cell) (Fig 6.2). Although *L. casei* Shirota does reduce both adhesion and invasion of Caco-2 cells by *S. enteritidis* 857 to more than 50%[32], the data of our pre- and co-incubation experiments suggest that this reduction does not benefit from *S. enteritidis* 857-induced IL-8 (Fig 6.2, 6.3). This could be due to the remaining number of *S. enteritidis* 857 invading the cells. Even the lowest number of *S. enteritidis* 857 (1 Se/cell) induced significant production of IL-8 (Fig 6.2). By contrast, exposure of Caco-2 cells to *S. enteritidis* 857 pre-treated with the SCS-LcS or SCS-Lp resulted in a marked inhibition of IL-8 synthesis (Fig 6.4) and in agar plate diffusion tests lactobacilli SCS inhibited the growth of *S. enteritidis* 857 (Table 6.1). The inhibition appeared to be specific to the SCS, since MRS or DMEM at pH 4.5 and 7.4 and lactic acid (100 mM), had no effect on growth (data

not shown), but revealed an intrinsic significant effect on IL-8 production (Fig 6.4). Several studies have implicated that the *Lactobacillus* microbial products mediate the beneficial effects in the intestinal epithelial cells[29,30]. The SCS from *L. acidophilus* reduces the cytoskeleton damage, inhibits the transcellular passage as well as the intracellular growth of the bacteria, and suppresses IL-8 production by *S. typhimurium* infecting enterocyte-like Caco-2 cells[30]. SCS from another *Lactobacillus*, *L. casei* GG, impede the invasion of Caco-2 cells without modifying the bacterial viability[29]. The mechanism underlying these events seem to be mediated through an antimicrobial substance present in the SCS of lactobacilli[33] that act directly against the pathogen. By inhibiting adhesion of *Salmonella* to Caco-2 cells[34], this substance is thought to account for inhibition of IL-8 production by Caco-2 cells via blockade of the adhesion-dependent *S. typhimurium*-induced cytokine production[30].

The beneficial effects of probiotics in host defence against infection have been suggested to include anti-inflammatory properties involving signalling with the gastrointestinal epithelium and with mucosal regulatory T-cells[35]. Probiotics may also counterbalance epithelial responses to invasive bacteria by regulating the cytokine transcription factors[36]. In the latter mechanism of action, Hsps might play an essential role through their ability to interfere with cytokine production in intestinal epithelial cells[6]. To the best of our knowledge, no data are available to explain the ability of lactobacilli SCS to express Hsp70 in the intestinal epithelial cells. An extensive experimental investigation conducted in the animal models, showed that *Lactobacillus* microbial components produced a marked long-term protection against ischemised rat heart. This effect was attributed to an activation of the cellular defence system manifested by overexpression of Hsp70[12]. We have demonstrated previously the ability of enterocyte-like Caco-2 cells to produce the protective Hsp70 at various conditions[4,37]. In this study, we show that *L. casei* Shirota and *L. plantarum* 299v are capable of inducing Hsp70 expression in the enterocyte-like Caco-2 cells (Fig 6.5). This response is specific to their SCS since the bacteria, MRS broth, or variations in pH could not induce the Hsp70 expression. Because production of Hsp70 is a protective response[4,6], we suggest that one mechanism for the beneficial attributes of *L. casei* Shirota and *L. plantarum* 299v is the ability of their antimicrobial products to induce expression of Hsp70. The induced Hsp70 could function in the stabilization of the cytoskeleton of intestinal epithelial cells after being distorted during adhesion and invasion by *Salmonella*[38,39] that could in turn, hamper further *Salmonella* invasion. In addition, based on the fact that SCS from *L. casei* GG impede invasion of Caco-2 cells by *S. typhimurium* without altering their viability[29], expression of Hsp70 could protect cells against the viable intracellular bacteria and/or the pro-inflammatory cytokines they produce. During the inflammatory response,

expression of several genes including IL-8 biosynthesis is regulated by the nuclear factor kappa B (NF-κB) and the activator protein (AP)-1 transcription factors. The anti-inflammatory role of Hsps stems from the blockade of the NF-κB transcription factors and the modulation of the AP-1 activities. These effects involve inhibition of inhibitory kappa B degradation and the AP-1 inflammatory responses mediated by p38 of the mitogen activated protein kinase pathway[6]. Indeed, expression of Hsp70 by lactobacilli SCS could be another mechanism imparted by *Lactobacillus* spp against the intestinal diseases associated with elevated IL-8 levels.

In summary, we have shown the biological significance of *L. casei* Shirota and *L. plantarum* 299v against *S. enteritidis* 857 infecting the human enterocyte-like Caco-2 cells. The data presented in this study show that it is not the bacteria themselves, but rather their antimicrobial products, that mediate the beneficial effects of *L. casei* Shirota and *L. plantarum* 299v. In particular, we report that the secreted antimicrobial products (SCS-LcS, SCS-Lp) inhibit the *S. enteritidis* 857-induced IL-8 production, the bacterial growth in agar plate diffusion tests and induce the expression of Hsp70. These activities may have *in vivo* ramifications in the improvement of gastroenteritis induced by *Salmonella* infections. Future studies focusing on the isolation and purification of the bacteriocin-like components of the lactobacilli SCS could help to explore the exact mechanisms imparted by *Lactobacillus* spp to the host defence.

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References

1. McCormick BA, Parkos CA, Colgan SP, Carnes DK, Madara JL. 1998. Apical secretion of a pathogen-elicited epithelial chemoattractant activity in response to surface colonization of intestinal epithelia by *Salmonella typhimurium*. *J Immunol* 160: 455-466.
2. Lee CA, Silva M, Siber AM, Kelly AJ, Galyov E, McCormick BA. 2000. A secreted *Salmonella* protein induces a proinflammatory response in epithelial cells, which promotes neutrophil migration. *Proc Natl Acad Sci U S A* 97: 12283-12288.
3. 3Greenberg DE, Jiang ZD, Steffen R, Verenker MP, DuPont HL. 2002. Markers of inflammation in bacterial diarrhea among travelers, with a focus on enteroaggregative *Escherichia coli* pathogenicity. *J Infect Dis* 185: 944-949.
4. Ovelgönne JH, Koninkx JFJG, Pusztai A, Bardocz S, Kok W, Ewen SW, Hendriks HG, van Dijk JE. 2000. Decreased levels of heat shock proteins in gut epithelial cells after exposure to plant lectins. *Gut* 46: 679-687.

5. Prohászka Z, Singh M, Nagy K, Kiss E, Lakos G, Duba J, Füst G. 2002. Heat shock protein 70 is a potent activator of the human complement system. *Cell Stress Chaperones* 7: 17–22.
6. Malago JJ, Koninkx JF, van Dijk JE. 2002. The heat shock response and cytoprotection of the intestinal epithelium. *Cell Stress Chaperones* 7: 191-199.
7. Schrezenmeir J, de Vrese M. 2001. Probiotics, prebiotics and synbiotics – approaching a definition. *Am J Clin Nutr* 73: Suppl 2, 361s-364s.
8. Fuller R. 1989. Probiotics in man and animals. *J Appl Bacteriol* 66: 365-378.
9. Brassart D, Schiffrin EJ. 1997. The use of probiotics to reinforce mucosal defense mechanisms. *Trends Food Sci Technol* 9: 321-326.
10. Reid G, Howard J, Gan BS. 2001. Can bacterial interference prevent infection? *Trends Microbiol* 9: 424-428.
11. Wallace TD, Bradley S, Buckley ND, Green-Johnson JM. 2003. Interactions of lactic acid bacteria with human intestinal epithelial cells: effects on cytokine production. *J Food Prot* 66: 466-472.
12. Oxman T, Shapira M, Diver A, Klein R, Avazov N, Rabinowitz. 2000. A new method of long-term preventive cardioprotection using *Lactobacillus*. *Am J Physiol Heart Circ Physiol* 278: H1717-H1724.
13. Sugita T, Togawa M. 1994. Efficacy of lactobacillus preparation biolactis powder in children with rotavirus enteritis. *Jap J Ped* 47: 2755-2762.
14. Campieri M, Rizzello F, Venturi A, Poggioli G, Ugolini F, Helwig U, Amadini C, Romboli E, Gionchetti P. 2000. Combination of antibiotic and probiotic treatment is efficacious in prophylaxis of post-operative recurrence of Crohn's disease: a randomised controlled study vs mesalamine. *Gastroenterology* 118: G4179.
15. Gionchetti P, Rizzello F, Venturi A, Brigidi P, Matteuzzi D, Bazzocchi G, Poggioli G, Miglioli M, Campieri M. 2000. Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. *Gastroenterology* 119: 305-309.
16. Hecht G, Savkovic SD. Effector role of epithelia in inflammation: interaction with bacteria. 1997. *Aliment Pharmacol Ther* Suppl 3:64-69.
17. Hata K, Andoh A, Sato H, Araki Y, Tanaka M, Tsujikawa T, Fujiyama Y, Bamba T. 2001. Sequential changes in luminal microflora and mucosal cytokine expression during developing of colitis in HLA-B27/beta2-microglobulin transgenic rats. *Scand J Gastroenterol* 36: 1185-1192.
18. Harig JM, Soergel KH, Komorowski RA, Wood CM. 1989. Treatment of diversion colitis with short chain fatty acid irrigation. *N Engl J Med* 320: 23-28.
19. O'Morain C, Segal AW, Levi AJ. 1982. Elemental diet as primary treatment of acute Crohn's disease: A controlled trial. *BMJ* 288: 1859-1862.
20. Sanderson IR. 1997. Diet and gut inflammation. *Curr Opin Gastroenterol* 13: 518-524.
21. Casellas F, Borruel N, Papo M, Guarner F, Antolin M, Videla S, Malagelada JR. 1998. Antiinflammatory effects of enterically coated amoxicillin-clavulanic acid in active ulcerative colitis. *Inflamm Bowel Dis* 4: 1-5.
22. Vimal DB, Khullar M, Gupta S, Ganguly NK. 2000. Intestinal mucins: the binding sites for *Salmonella typhimurium*. *Mol Cell Biochem* 204: 107-117.
23. Salminen S, Bouley C, Boutron-Ruault MC, Cummings JH, Franck A, Gibson GR, Isolauri E, Moreau MC, Roberfroid M, Rowland I. 1998. Functional food science and gastrointestinal physiology and function. *Br J Nutr* 80 Suppl 1: S147-71.
24. Lee YK, Namoto K, Salminen S, Gorbach SJ. 1999. Handbook of probiotics. New York: John Wiley & Sons.
25. Koninkx JFJG, Hendriks HGCJM, van Rossum JMA, van den Ingh TSGAM, Mouwen JMVM. 1992. Interaction of legume lectins with the cellular metabolism of differentiated Caco-2 cells. *Gastroenterology* 102: 1516-1523.

26. Koninkx JFJG. 1995. Enterocyte-like Caco-2 cells as a tool to study lectin interaction. In: *Lectins: biomedical perspectives*, ed Pusztai A, Bardocz S. Taylor and Francis, London, 81-101.
27. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic acid. *Anal Biochem* 159: 76-85.
28. Classen E, van Vinsen R, Posko M, Boersma WAU. 1995. New and safe 'oral' live vaccines based on *Lactobacillus. Adv Exp Med Biol* 371B: 1553-1558.
29. Hudault S, Liévin V, Bernet-Camard MF, Servin AL. 1997. Antagonistic activity exerted in vitro and in vivo by *Lactobacillus casei* (strain GG) against *Salmonella typhimurium* C5 infection. *Appl Environ Microbiol* 63: 513-518.
30. Coconnier MH, Liévin V, Lorrot M, Servin AL. 2000. Antagonistic activity of *Lactobacillus acidophilus* LB against intracellular *Salmonella enterica* serovar Typhimurium infecting human enterocyte-like Caco-2/TC-7 cells. *Appl Environ Microbiol* 64: 4573-4580.
31. Eckmann, L., Jung, H.H., Schürer-Maly, C., Panja, A., Morzycka-Wroblewska, E., and Kagnoff, M.F. 1993. Differential cytokine expression by human intestinal epithelial cell lines: regulated expression of interleukin 8. *Gastroenterology* 105: 1689-1697.
32. Hendriks H, de Jonge R, van Asten F, Koninkx J, Swennenhuis J, van Dijk J. 2001. Adherence and invasion of *Salmonella entritidis* 857 to Caco-2 cells decreases after simultaneous incubation with *Lactobacillus casei* Shirota. In: *Typhoid fever and other salmonellosis*, ed Ou JT, Chiu CH, Chu C. Taipei, Taiwan, 135-141.
33. Silva M, Jacobus NV, Deneke C, Gorbach SL. 1987. Antimicrobial substance from a human Lactobacillus strain. *Antimicrob Agents Chemother* 31: 1231-1233.
34. Coconnier MH, Liévin V, Bernet-Camard MF, Hudault S, Servin AL. 1997. Antibacterial effect of the adhering human *Lactobacillus acidophilus* strain LB. *Antimicrob Agents Chemother* 41: 1046-1052.
35. Shanahan F. 2000. Probiotics and inflammatory bowel disease: is there a scientific rationale? *Inflamm Bowel Dis* 6: 107-115.
36. Neish AS, Gewirtz AT, Zeng H, Young AN, Hobert ME, Karmali V, Rao AS, Madara JL. 2000. Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination. *Science* 289: 1560-1563.
37. Malago JJ, Koninkx JFJG, Ovelgönne HH, van Asten FJAM, Swennenhuis JF, van Dijk JE. 2003. Expression levels of heat shock proteins in enterocyte-like Caco-2 cells after exposure to *Salmonella enteritidis. Cell Stress Chaperones*, in press.
38. Jepson MA, Colares-Buzato CB, Clark MA, Hirst BH, Simmons NL. 1995. Rapid disruption of epithelial barrier function by Salmonella typhimurium is associated with structural modification of intercellular junctions. *Infect Immun* 63: 356-359.
39. Jepson MA, Lang TF, Reed KA, Simmons NL. 1996. Evidence for a rapid, direct effect on epithelial monolayer integrity and transepithelial transport in response to *Salmonella* invasion. *Eur J Physiol* 432: 225-233.

7

SUMMARY AND CONCLUDING REMARKS

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Heat shock proteins (Hsps), first observed because they are preferentially synthesized by organisms exposed to heat or other physiological stress, are known to be also present constitutively in certain tissues. They are divided in several families according to their size, structure and function. Hsps exist in all organisms from bacteria to humans and they are among the most conserved proteins known. As a result of their synthesis, Hsps gain the organism tolerance to insult, a phenomenon termed induced thermotolerance or stress tolerance. Hsps are also essential for the well being of unstressed cells by facilitating *in vivo* fates of many proteins such as folding, oligomeric assembly, transport to a particular subcellular compartment, or controlled switching between the active and inactive conformations.

The objective of this study was to investigate the role of the Hsps in the cytoprotection of the intestinal epithelium under various conditions ranging from exposure to normal physiological intestinal dietary products to pathogenic bacteria. Parameters on growth, differentiation, inflammatory cytokine regulation, invasion by pathogenic bacteria and involvement of the beneficial gut flora were assessed in enterocyte-like Caco-2 cells.

This human colon carcinoma cell line Caco-2 has been used throughout our experiments. Whereas undifferentiated 5-day-old Caco-2 cells can be regarded as the *in vitro* counterpart of enterocytes in the crypts, fully differentiated 19-day-old Caco-2 cells represent the *in vitro* counterpart of enterocytes on the villus. Fully differentiated Caco-2 cells exhibit properties of small intestinal enterocytes both structurally and functionally. Microvilli are present on the apical surface of the differentiated cells and tight junctions achieve the polarity of the Caco-2 cells. The high level of activity of brush border-associated enzymes including alkaline phosphatase, aminopeptidase and sucrase-isomaltase are characteristic for the functional differentiation of these cells.

A comprehensive review on the interference of Hsps with the inflammatory cytokine production is presented in Chapter 2. This review uncovers the major inflammatory production pathways, i.e. the nuclear factor kappa B (NF-κB) and the mitogen activated protein kinase (MAPK) pathways, as well as their events that lead to cytokine production. It also explains how Hsps are produced and their interference with NF-κB and MAPK pathways to regulate inflammatory cytokine production. It is interesting to note that Hsps are also involved in the regulation of growth signals, a function that might have *in vivo* ramifications in the repair of damaged intestinal epithelium.

We established the growth, metabolism, differentiation and protection of enterocyte-like Caco-2 cells against pathogenic bacteria when grown in the presence or absence of the short-chain fatty acids formate, propionate and butyrate.

As described in Chapter 3, the short chain fatty acids affected the growth, the metabolism and the differentiation of the Caco-2 cells in a concentration- and carbon atom-related pattern. Formate was the most potent growth promoter while butyrate was inhibitory and propionate had an intermediate effect. Only propionate and butyrate induced markers of differentiation with butyrate being the most potent one. Since Hsps are involved in cellular growth and repair, protective assessment in this chapter focused on the significance of differentiation, in particular the induction of the transepithelial electrical resistance. An increase in the transepithelial electrical resistance indicates that the epithelial integrity is optimal and thus passage of substances across the epithelium is controlled. The increased transepithelial electrical resistance or reduced paracellular permeability could be a result of increased tight junction functions that may involve activation of genes coding for tight junction specific proteins. Our interest was to see whether a change in the transepithelial electrical resistance impedes the cellular invasion by pathogenic bacteria. Experiments of our study show that both propionate and butyrate induced an increase in the transepithelial electrical resistance in enterocyte-like Caco-2 cells. Although this finding is suggestive of an intact epithelium in terms of barrier integrity, the induced increase in the transepithelial electrical resistance did not protect the cells from *Salmonella enteritidis* invasion. Therefore, from these data the conclusion was drawn that for the induced transepithelial electrical resistance to confer protection, ample time is probably needed for the establishment of barrier integrity especially the role of tight junctions.

After observing the failure of short chain fatty acid-induced transepithelial electrical resistance to protect the cells against pathogenic *S. enteritidis* invasion, the experiments in Chapter 4 were designed to answer the question on how the short chain fatty acids impart the intestinal welfare. The experiments were conducted to specifically explore the effect of the most potent and abundant short chain fatty acid, butyrate, on Hsp70 and interleukin (IL)-8 production in the enterocyte-like Caco-2 cells. Substantial studies have linked butyrate deficiency with the development of ulcerative colitis, Crohn's disease and other forms of inflammatory bowel diseases characterized by elevated levels of IL-8. In addition, intracolonic infusion of butyrate enemas benefits these patients. However, the actual mechanism is incompletely understood. We hypothesized that Hsps might be involved in the butyrate-mediated alleviation of intestinal inflammations by modulating the IL-8 production. Since infection of intestinal epithelial cells by *S. enteritidis* leads to production of IL-8, we studied the effect of butyrate on the *S. enteritidis*-induced synthesis of IL-8 and whether this effect is mediated, at least in part, via production of Hsp70. Our results show that induction of Hsp70 by thermal stress inhibits the proinflammatory IL-8 production. In addition, butyrate, dose-

dependently, induces the expression of Hsp70 and modulates IL-8 production. It is therefore most likely that the expression of Hsp70 by butyrate accounts for the inhibition of IL-8 production. Since high intestinal IL-8 levels are characteristic of many intestinal inflammations, butyrate might impart its therapeutic significance by lowering these levels. Its mechanism could involve, at least in part, the production of Hsp70.

Through their adhesins, *Salmonella* species are capable of binding and invading mucosal barriers. As they do so, they cause structural lesions at the apical membrane of the cells and elicit severe disruptions in the integrity of the epithelial monolayer. These changes are characterized by a rearrangement of actin filaments and the subsequent decrease in transepithelial electrical resistance and are sensitive to inhibitors of actin filament polymerization. Evidence is increasing to emphasize the relationship between Hsps and the cytoskeleton. Induction of Hsps has been shown to prevent actin depolymerization and to protect the integrity of the actin cytoskeleton against various injuries. In particular, Hsp70 and Hsp90 are reported to stabilize the actin filaments by cross-linking. This in turn, could prevent the membrane disruptions caused by pathogens like *S. enteritidis*. We therefore, investigated in Chapter 5 whether pathogenic *S. enteritidis* is capable of inducing Hsps in enterocyte-like Caco-2 cells. Our results in this chapter clearly show that live *S. enteritidis* induces the expression of Hsp70 and Hsp90 in crypt-like as well as villus-like Caco-2 cells. Killed bacteria, live bacteria incubated with chloramphenicol, or the bacterial endotoxin of *S. enteritidis* (LPS^{Se}) itself appeared to be unable to induce the stress response. This alludes that the live *S. enteritids* itself is stressful to the cells and not its endotoxin. Further, our experiments clearly showed dose-related changes in the transepithelial electrical resistance that were suggestive of a loss of mucosal integrity. On the basis of these findings, it was reasonable to suggest that actin filaments are recruited and remodelled by *S. enteritidis* so as to enter the Caco-2 cells. We concluded in this chapter that the process of invasion of the intestinal epithelial cells by *S. enteritidis* could be responsible to trigger the Hsp response. If this is so, then the induced Hsps might be protective against the insulting stimulus, which in this case, is the bacterial invasion. However, the constitutive levels of Hsps in the cells appeared to be insufficient to protect the cells from invasion by *S. enteritids*. It is therefore reasonable to suggest that the induction of Hsps following bacterial exposure is developed too late to render protection. To protect the cells against bacterial invasion they most likely would benefit from previously induced high levels of Hsps. Moreover, the biological significance of the synthesized Hsps might be diverse.

The protective potential of the lactic acid bacteria that constitute a considerable proportion of the normal gut flora of the human gastrointestinal tract

was studied and findings have been described in Chapter 6. Selected strains from this flora exert a positive influence on host health or physiology when ingested. *Lactobacillus casei* Shirota and *L. plantarum* 299v have been implicated in the treatment of specific local intestinal inflammations like ulcerative and *Clostridium difficile* colitis, Crohn's disease, pouchitis, and diarrheagenic illnesses. Most, if not all, of these disorders are characterized by high levels of IL-8 production. Because patients significantly benefit from interventions that decrease the IL-8 levels, we investigated the effect of *L. casei* Shirota and *L. plantarum* 299v and their spent culture supernatants on Hsp70 and IL-8 synthesis by Caco-2 cells. The non-specific binding *L. casei* Shirota and the mannose-specific *L. plantarum* 299v had little effect on the production of IL-8 in Caco-2 cells either alone or in combination with the pathogenic *S. enteritidis* 857. When Caco-2 cells were either pre-incubated or co-incubated with the lactobacilli and *S. enteritidis* 857, the pattern of IL-8 production observed was similar to that exhibited by Caco-2 cells exposed to *S. enteritidis* 857 alone. In addition, the lactobacilli did not induce the expression of Hsp70. These observations imply that the lactobacilli affect neither *S. enteritidis* 857-induced production of IL-8 nor Hsp70 expression. On the contrary, exposure of the cells to *S. enteritidis* 857 pretreated with the spent culture supernatants of lactobacilli resulted in a marked inhibition of the *S. enteritidis* 857-induced IL-8 production and stimulation of the Hsp70 synthesis. Moreover, the spent culture supernatants inhibited the growth of *S. enteritidis* 857 as evaluated by the agar plate diffusion test. In conclusion, our data suggest that the beneficial effect of lactobacilli is mediated via an antimicrobial product present in the spent culture supernatants either directly against the pathogens or indirectly through the synthesis of Hsp70.

Both intracolonic infusion of butyrate enemas and oral administration of *Lactobacillus* spp as probiotics are gaining importance in the treatment of various intestinal inflammations. Their beneficial effects in host defence against infection include anti-inflammatory properties involving signalling with the gastrointestinal epithelium and with mucosal regulatory immune cells. They also counterbalance the epithelial responses to invasive bacteria by regulating the cytokine transcription factors. The mechanism of action appears to involve Hsp expression. Since the evolutionary conserved Hsps have diverse protective role, incorporation of short chain fatty acids, such as butyrate, or lactobacilli and/or their products to diets as food additives, could secondarily benefit humans and animals against various intestinal disorders. The benefit stems from the ability of these agents to induce Hsp synthesis in intestinal epithelial cells.

Our findings from these studies show that the intestinal epithelial cells are triggered to synthesize Hsps under various conditions such as thermal stress, *S. enteritidis* 857 infection, exposure of the cells to products of the normal intestinal

flora (butyrate) and *S. enteritidis* 857 pretreated with the spent culture supernatants of lactobacilli. These Hsps are, however, limited in their scope of protection. For instance, while they interfere with the cellular growth and stabilize the intestinal barrier and cell cytoskeleton integrity, it appears that the increased levels of Hsps inside the cells do not protect them against pathogenic invasion. By acting as chaperones through protein-protein interaction, Hsps modulate the inflammatory cykokine production to curb the intestinal inflammations. The potential of butyrate and the spent culture supernatants of lactobacilli to induce Hsps is an important finding and suggests involvement of the Hsps in the improvement of various intestinal inflammations. Since the heat shock results in the synthesis of Hsps that accounts for the suppression of IL-8 production, the improvement of various intestinal inflammations (that are characterized by high IL-8 levels) by butyrate or lactobacilli might be mediated, at least in part, via the synthesis of Hsps. This study suggests that a strong link exists between Hsps and the improvement of such conditions. It should be borne in mind that Hsps are limited in their scope of protection. Other protective mechanisms existing in the intestinal epithelium may not involve the production of Hsps. Thus, the limited Hsp scope of the protection of the intestinal epithelial cells is substantiated by other protective phenomena to maintain the normal intestinal structure and function. Further studies are needed to explore more the biological significance of the Hsp-mediated improvement of the barrier integrity and the modulation of other pro- and anti-inflammatory cytokines.

Samenvatting en conclusies

Van heat shock eiwitten (Hsps) die voor het eerst ontdekt werden in cellen na blootstelling aan hitte of andere fysiologische stress omstandigheden, is bekend dat ze in bepaalde weefsels ook constitutief kunnen voorkomen. Zij worden naar grootte, structuur en functie onderverdeeld in meerdere families. Hsps komen in alle organismen van bacteriën tot mens voor en zijn met enkele andere eiwitten evolutionair gezien de meest conservatieve eiwitten die er bestaan. Na hun synthese bieden Hsps bescherming aan organismen tegen beschadiging, een fenomeen dat geïnduceerde thermotolerantie of stress tolerantie wordt genoemd. Hsps zijn ook belangrijk in de niet gestresste cel: ze hebben een belangrijke functie bij het vouwen van eiwitten, de oligomere assemblage ervan, bij het transport van eiwitten door de cel en bij het schakelen tussen de actieve en inactieve conformatie van eiwitten.

In dit proefschrift is de rol van Hsps onderzocht bij de bescherming van het darmepitheel onder verschillende omstandigheden variërend van blootstelling aan normale fysiologische voedingsproducten tot pathogene bacteriën. Onder deze condities is in een darmepitheelcellijn (Caco-2 cellen) het effect op de groei, de differentiatie, de regulatie van ontsteking gerelateerde cytokines, de invasie van pathogene bacteriën en de invloed van een beschermende darmflora onderzocht.
De humane colon carcinoma cellijn Caco-2 is bij al onze experimenten gebruikt. Terwijl ongedifferentieerde 5 dagen oude Caco-2 cellen beschouwd kunnen worden als de *in vitro* tegenhanger van de enterocyten in de crypte, vertegenwoordigen gedifferentieerde 19 dagen oude Caco-2 cellen de *in vitro* tegenhanger van de enterocyten op de villus. Volledig gedifferentieerde Caco-2 cellen laten zowel morfologische als functionele eigenschappen van dunne-darm enterocyten zien. Microvilli zijn aanwezig op het apicale oppervlak van de gedifferentieerde cellen en tight junctions geven de Caco-2 cellen hun polariteit. De hoge activiteit van de borstelzoom geassocieerde enzymen zoals alkalische fosfatase, aminopeptidase en sucrase-isomaltase zijn kenmerkend voor de functionele differentiatie van deze cellen.

In hoofdstuk 2 is een uitgebreide samenvatting te vinden over effecten van Hsps op de synthese van cytokines bij een ontstekingsproces. Dit overzichtsartikel behandelt de belangrijkste metabole wegen leidend tot de synthese van ontstekingsproducten, zoals de nuclear factor kappa B (NF-κB) en het mitogeen geactiveerde protein kinase (MAPK) en de gebeurtenissen die leiden tot de synthese van cytokines. Wanneer Hsps worden gesynthetiseerd en hoe deze synthese wordt beïnvloed door de metabole wegen van NF-κB en MAPK bij de regulatie ervan, wordt ook duidelijk gemaakt. Een ander belangrijk aspect van

Hsps is dat ze betrokken zijn bij de regulatie van groeisignalen, een functie die *in vivo* zou kunnen bijdragen bij het herstel van het beschadigd darmepitheel.

In de aanwezigheid respectievelijk afwezigheid van vetzuren met korte koolstofketens (SCFAs) zoals formaat, propionaat en butyraat is het effect op de groei, het metabolisme, de differentiatie en de bescherming van Caco-2 cellen tegen pathogene bacteriën onderzocht. In hoofdstuk 3 is een dosis en koolstofketen lengte gerelateerd patroon aangetoond in de groei, het metabolisme en de differentiatie. Formaat blijkt de meest krachtige groei promotor te zijn, terwijl butyraat een remmend en propionaat een intermediair effect laat zien. Alleen propionaat en butyraat waren in staat differentiatie markers te induceren. In dit opzicht was butyraat het meest effectief. Omdat Hsps betrokken zijn bij de groei en het herstel van cellen, is in dit hoofdstuk vooral het beschermende aspect van de differentiatie vastgesteld, in het bijzonder gericht op de inductie van de transepitheliale electrische weerstand. Een toename van de transepitheliale weerstand geeft aan, dat de epitheliale integriteit optimaal is en dat dus de passage van stoffen door het epitheel gecontroleerd wordt. De toegenomen transepitheliale electrische weerstand of gereduceerde paracellulaire permeabiliteit zou het gevolg kunnen zijn van een verbeterd functioneren van de tight junctions. De activering van genen, die coderen voor tight junction specifieke eiwitten, is hier waarschijnlijk bij betrokken. In onze experimenten is te zien, dat zowel propionaat als butyraat een toename in de transepitheliale electrische weerstand van de Caco-2 cellen induceert. Hoewel dit resultaat suggereert dat er sprake is van een intact epitheel, uitgedrukt in barrière integriteit, blijkt de geïnduceerde toename van de transepitheliale electrische weerstand niet in staat te zijn de cellen te beschermen tegen een invasie door *Salmonella enteritidis*. Uit deze resultaten kan daarom de conclusie getrokken worden, dat een eventuele beschermende werking gelieerd aan de geïnduceerde transepitheliale electrische weerstand pas na langere tijd effect sorteert. Deze tijd is nodig om de integriteit van de barrière, waarbij de tight junctions een belangrijke rol spelen, te optimaliseren.

Omdat een door kortketen vetzuren geïnduceerde toename in de transepitheliale electrische weerstand niet leidde tot extra bescherming van cellen tegen de invasie van *Salmonella enteritidis* 857, zijn in hoofdstuk 4 experimenten uitgevoerd die tot doel hadden antwoord te geven op de vraag of butyraat op een andere manier de conditie van de darm kan verbeteren. De experimenten werden zodanig uitgevoerd, dat specifiek het effect van het meest effectieve en meest voorkomende kortketen vetzuur, butyraat, op de Hsp70 en IL-8 productie in Caco-2 cellen onderzocht kon worden. In een groot aantal studies wordt een koppeling gelegd tussen butyraat deficiënties en de ontwikkeling van ulceratieve colitis, de ziekte van Crohn en andere vormen van ontstekingsziekten van de darm. Kenmerkend voor deze darmziekten is een verhoogde IL-8 concentratie.

Bovendien blijkt, dat toediening van butyraat via een klisteerspuit in het colon (butyraat enemas) een heilzame werking op een aantal van deze patiënten heeft. Het werkingsmechanisme is nog niet volledig bekend. De betrokkenheid van Hsps in deze is nog steeds onduidelijk. Onze werkhypothese is dat Hsps via modulatie van de IL-8 productie betrokken zijn bij de butyraat-gerelateerde verlichting van de ontstekingen in de darm. Omdat een infectie van darmepitheelcellen door *S. enteritidis* de productie van IL-8 tot gevolg heeft, hebben we het effect van butyraat op de *S. enteritidis* geïnduceerde synthese van IL-8 onderzocht. Bovendien is nagegaan of dit effect tenminste voor een deel gestuurd wordt via de productie van Hsp70. De resultaten laten zien dat inductie van Hsps door hyperthermie de synthese van het proinflammatoire IL-8 remt. Bovendien induceert butyraat dosis-afhankelijk de expressie van Hsp70 en moduleert het de synthese van IL-8. Het is dan ook waarschijnlijk, dat de door butyraat-geïnduceerde expressie van Hsp70 de remming van de productie van IL-8 verklaart. Omdat hoge IL-8 concentraties kenmerkend zijn voor ontstekingen in de darm, vindt de therapeutische betekenis van butyraat zijn oorsprong in een verlaging van deze concentraties. De productie van Hsp70 is waarschijnlijk een deel van het hierbij betrokken werkingsmechanisme.

Via hun adhesines zijn Salmonella species in staat te binden aan mucosale barrières en deze te invaderen. Tijdens dit proces veroorzaken ze structurele laesies aan de apicale membraan van de cellen en vinden er ernstige verstoringen plaats in de integriteit van de epitheliale monolaag. Karakteristiek voor deze veranderingen zijn een reorganisatie van de actine filamenten en een daarop volgende afname van de transepitheliale electrische weerstand. Beide veranderingen zijn gevoelig voor inhibitoren van de actine filament polymerisatie. De hoeveelheid bewijsmateriaal is groeiende waarin de relatie tussen Hsps en het cytoskelet wordt benadrukt. Het is bekend dat na de inductie van Hsps de depolymerisatie van de actine filamenten is geblokkeerd en dat de integriteit van het actine cytoskelet de cel bescherming biedt tegen een groot aantal beschadigingen. Vooral van Hsp70 en Hsp90 is bekend, dat ze de actine filamenten door cross-linking stabiliseren. Membraanbeschadigingen veroorzaakt door pathogenen zoals *S. enteritidis* zouden door cross-linking voorkomen kunnen worden. In hoofdstuk 5 is daarom onderzocht of *S. enteritidis* in staat is Hsps te induceren in de darmepitheelcellijn Caco-2. De resultaten in dit hoofdstuk laten duidelijk zien dat vitale *S. enteritidis* bacteriën de expressie van Hsp70 en Hsp90 induceren in zowel crypt-like als villus-like Caco-2 cellen. Dode bacteriën, levende bacteriën na incubatie met chloramphenicol en het endotoxine van *S. enteritidis* (LPS^{Se}) blijken echter niet in staat te zijn de stress respons te induceren. Met andere woorden *S. enteritidis* zelf is de stressfactor voor de cellen en niet diens endotoxine. Verder laten onze experimenten duidelijk dosis-gerelateerde veranderingen in de transepitheliale electrische weerstand zien, die

een verlies van de mucosale integriteit suggereren. Met deze resultaten als uitgangspunt is het dan ook redelijk om te veronderstellen, dat de actine filamenten door *S. enteritidis* gerekruteerd en zodanig omgevormd worden dat invasie van Caco-2 cellen mogelijk wordt. We hebben in dit hoofdstuk de conclusie getrokken, dat het invasie-proces van de darmepitheelcellen door *S. enteritidis* de trigger tot de Hsp respons zou kunnen zijn. Als dit zo is, dan zouden de geïnduceerde Hsps bescherming kunnen bieden tegen de schade toebrengende prikkel. In dit geval is dat dus de invasie van bacteriën. De constitutieve Hsp concentraties bleken echter niet in staat te zijn de cellen te beschermen tegen een invasie door *S. enteritidis*. Het is dan ook redelijk te veronderstellen, dat in ons experimentele model de inductie van Hsps na blootstelling aan bacteriën te laat komt om voor bescherming te kunnen zorgen. Om de cellen tegen een bacteriële invasie te beschermen zouden ze hoogstwaarschijnlijk baat vinden bij hoge concentraties Hsps, wanneer die reeds voor het moment van blootstelling aanwezig zijn. De biologische betekenis van de gesynthetiseerde Hsps zou bovendien wel eens meer divers kunnen zijn.

De beschermende potentie van de melkzuurbacteriën, die een zeer belangrijk deel uitmaken van de normale darmflora in het maagdarmkanaal van de mens, is onderzocht en in hoofdstuk 6 staan de resultaten vermeld. Bepaalde stammen uit deze flora hebben na inname een positief effect op de gezondheid en fysiologie van de gastheer. *Lactobacillus casei* Shirota en *L. plantarum* 299v worden bijvoorbeeld toegepast bij de behandeling van specifieke locale darmontstekingen zoals ulceratieve colitis en door *Clostridium difficile* veroorzaakte colitis, de ziekte van Crohn, pouchitis en ziekten die met diarree gepaard gaan. Karakteristiek voor het merendeel van deze kwalen zijn de hoge IL-8 concentraties. Omdat de patiënten significant baat vinden bij behandelingen, die de IL-8 concentraties verlagen, hebben we het effect van *L. casei* Shirota, *L. plantarum* 299v en diens in de bouillon aanwezige secretie-producten (spent culture supernatants: SCS) onderzocht op de Hsp70 en IL-8 synthese van Caco-2 cellen. De niet-specifieke binding van *L. casei* Shirota en de mannose-specifieke binding van *L. plantarum* 299v hadden weinig effect op de productie van IL-8 in de Caco-2 cellen. Dit werd zowel waargenomen na incubatie van de cellen met de lactobacilli apart, maar ook in combinatie met de pathogene bacterie *S. enteritidis* 857. Na preincubatie of co-incubatie van Caco-2 cellen met lactobacilli en *S. enteritidis* 857 bleek het IL-8 patroon gelijk te zijn aan dat van cellen die alleen waren blootgesteld aan *S. enteritidis* 857. Bovendien bleken de lactobacilli niet in staat te zijn de expressie van Hsps te induceren. Deze waarnemingen betekenen, dat de lactobacilli geen invloed hebben op de door *S. enteritidis* geïnduceerde expressie van IL-8 en Hsp70. Blootstelling van de cellen aan de met SCS-voorbehandelde *S. enteritidis* 857 bacteriën resulteerde in een opvallende remming van de door *S. enteritidis* 857 geïnduceerde IL-8 productie en de inductie van de

Hsp synthese. Bovendien bleek uit een agarplaat diffusie test dat de in de bouillon aanwezige secretie-producten (spent cell supernatants) de groei van *S. enteritidis* 857 remden. Samenvattend kan gesteld worden dat het heilzame effect van de lactobacilli veroorzaakt wordt door een antimicrobieel product in de bouillon dat of direct tegen het pathogeen gericht is of indirect werkt via de synthese van Hsp70.

Zowel de toediening van butyraat via een klisteerspuit in het colon als de orale toediening van het probioticum *Lactobacillus* spp worden steeds belangrijker in de behandeling van verschillende ontstekingen in de darm. Bij de bescherming van de gastheer tegen infecties zijn het de anti-inflammatoire eigenschappen van butyraat en lactobacilli die een heilzaam effect sorteren. Het maagdarmepitheel en de mucosale regulatoire immuun cellen worden via signaaltransductie hierover geïnformeerd. Zij compenseren ook de epitheliale respons bij de invasie van bacteriën door de cytokine trancriptie factor te reguleren. De expressie van Hsps blijkt een onderdeel van het werkingsmechanisme te zijn. Omdat evolutionair gezien deze meest conservatieve eiwitten verschillende beschermende functies uitoefenen, is het zeer wel mogelijk, dat de toediening van kortketen vetzuren, zoals butyraat, lactobacilli of hun microbiële producten aan het dieet van mens en dier extra heilzaam werkt bij een verscheidenheid aan darmziekten. De heilzame werking ervan vindt zijn oorsprong in het vermogen van deze agentia de Hsp synthese in de darmepitheelcellen te induceren.

De resultaten van ons onderzoek laten zien dat darmepitheelcellen onder verschillende condities zoals stress door hyperthermie, infectie met *S. enteritidis* 857, blootstelling van de cellen aan producten van de normale darmflora (butyraat) en na incubatie van met het SCS van lactobacilli voorbehandelde *S. enteritidis* 857 worden aangezet tot de synthese van Hsps. De draagwijdte met betrekking tot de bescherming die deze Hsps bieden, is echter beperkt. Een voorbeeld hiervan is dat de Hsps enerzijds invloed hebben op de cellulaire groei, de darmbarrière en het cellulaire cytoskelet stabiliseren en anderzijds zelfs bij verhoogde Hsp concentraties in de cel geen bescherming bieden tegen een invasie van pathogenen. In hun functie van chaperones via eiwit-eiwit interacties moduleren de Hsps de productie van de inflammatoire cytokines, waardoor zij kunnen bijdragen aan het bedwingen van ontstekingen in de darm. De Hsp inducerende potentie van butyraat en van het in de bouillon aanwezige anti-microbiële product van de lactobacilli is een belangrijke ontdekking, die de betrokkenheid van Hsps suggereert bij de verbetering van verschillende ontstekingen in de darm. Omdat een heat shock leidt tot de synthese van Hsps, die op hun beurt de IL-8 productie remmen, zou een verbetering van darmontstekingen (hoge IL-8 zijn hierbij kenmerkend) voor een deel toegeschreven kunnen worden aan de synthese van Hsps. Dit onderzoek suggereert een sterke correlatie tussen Hsps enerzijds en de verbetering van darminfecties anderzijds. Het blijft echter belangrijk zich te realiseren, dat Hsps

beperkt zijn in hun bijdrage aan bescherming. Bij andere beschermende mechanismen, die ook in het darmepitheel aanwezig zijn, is de productie van Hsps niet vanzelfsprekend. Met andere woorden, de beperkte potentie van Hsps bij de bescherming van darmepitheelcellen wordt versterkt door andere beschermende mechanismen die tot doel hebben de normale structuur en functie van de darm te behouden. Om ons inzicht verder te verdiepen met betrekking tot de biologische betekenis van de door Hsp gemedieerde verbetering van de darmintegriteit en de modulatie van andere pro- en anti-inflammatoire cytokines is meer onderzoek nodig.

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With Very Best Wishes

Curriculum vitae

Joshua Joseph Malago was born on the 5th of September 1969 in Ngudu, Kwimba, Mwanza, Tanzania. He obtained his secondary education at Kilosa Secondary School from January 1985 to November 1988 (ordinary level) and at Kibaha Secondary School from July 1989 to May 1991 (advanced level). From September 1992 to July 1997 he pursued a degree course in Veterinary Medicine at Sokoine University of Agriculture (SUA), Morogoro, Tanzania and was awarded a Bachelor of Veterinary Medicine degree in November 1997. He then joined Utrecht University in September 1998 for a Master of Science in Animal Pathology degree course organized by the Department of Pathology of the Faculty of Veterinary Medicine and graduated in August 2000. In August 2000 SUA employed him as an Assistant Lecturer in the Department of Veterinary Pathology, Faculty of Veterinary Medicine. He started his PhD program in June 2001 in the Department of Pathology, Faculty of Veterinary Medicine, Utrecht University. After successfully defending his PhD thesis, he will continue to work with the Department of Veterinary Pathology in Morogoro.