Decreased levels of heat shock proteins in gut epithelial cells after exposure to plant lectins

J H Ovelgönne, J F J G Koninkx, A Pusztai, S Bardocz, W Kok, S W B Ewen, H G C J M Hendriks, J E van Dijk

Abstract

Background—The enterocytes of the intestinal epithelium are regularly exposed to potentially harmful substances of dietary origin, such as lectins. Expression of heat shock proteins (HSPs) by this epithelium may be part of a protective mechanism developed by intestinal epithelial cells to deal with noxious components in the intestinal lumen.

Aim—To investigate if the lectins PHA, a lectin from kidney beans (Phaseolus vulgaris) and WGA, a lectin from wheat germ (Triticum aestivum) could modify the heat shock response in gut epithelial cells and to establish the extent of this effect.

Methods—Jejunal tissue sections from PHA and WGA fed rats were screened for expression of HSP70, HSP72, and HSP90 using monoclonal antibodies. Differentiated Caco-2 cells, the in vitro counterpart of villus enterocytes, were exposed to 100 µg/ml of PHA-E4, or WGA for 48 hours and investigated for changes in DNA and protein synthesis by double labelling with [3H]thymidine and L-[methyl-14C]methionine. The relative concentrations of HSP60, HSP70, HSP72, and HSP90 and binding protein (BiP) in these cells exposed to lectins were analysed by polyacrylamide gel electrophoresis and immunoblotting. To establish if lectin exposed differentiated Caco-2 cells were still capable of producing a heat shock response, these cells received a heat shock (40°C, 41°C, and 42°C) for one hour and were allowed to recover for six hours at 37°C. During heat shock and recovery periods, lectin exposure was continued.

Results—Constitutive levels of HSPs were measured in the intestinal cells of lactalbumin fed (control) rats, as may be expected from the function of this tissue. However, in PHA and WGA fed rats a marked decline in the binding of antibodies against several HSPs to the intestinal epithelium was found. These results were confirmed by in vitro experiments using differentiated Caco-2 cells exposed to PHA-E4, and WGA. However, after exposure to lectins, these cells were still capable of heat induced heat shock protein synthesis, and total protein synthesis was not impaired indicating specific inhibition of HSP70 synthesis in non-stressed cells.

Conclusions—we conclude that PHA and WGA decrease levels of stress proteins in rat gut and enterocyte-like Caco-2 cells, leaving these cells less well protected against the potentially harmful content of the gut lumen.

Keywords: gut; rat small intestine; Caco-2 cells; lectins; stress proteins; heat shock proteins; heat shock

Living cells have a highly conserved response to adverse changes in their environmental conditions, commonly referred to as the heat shock response or stress response. Apparently a defensive mechanism, this response is elicited by a variety of physical and chemical agents, including heat shock, oxidising agents, heavy metals, sulphydryl reagents, anoxia, and ethanol.1–7 The most obvious characteristics of the stress response are overall inhibition of protein synthesis and enhanced synthesis of molecular chaperones, more commonly known as heat shock proteins (HSPs) or stress proteins. Exposure of cells to heat shock and other stressors results in virtual shutdown of normal cellular protein synthesis, paralleled by a shift to high levels of HSP synthesis.8–9 It is considered that induction of HSPs in mammalian cells is transcriptionally regulated. Transcription is initiated by a heat shock transcription factor.7 10–13 This protein is thought to be constitutively present14 and kept in an inactive state by its association with HSP70 in its quaternary structure. Subsequently the heat shock factor trimerises, migrates to the nucleus, and activates the promoters of heat shock genes.15 In addition to transcriptional regulation, translational regulation has been described in insect cells16 and there is some evidence for this type of regulation in mammalian cells.17 18

A large body of evidence links the stress response to a consequent decrease in cellular sensitivity to stress. It has been demonstrated that thermotolerance is conferred by increased levels of HSPs. This has been observed under conditions where HSPs are induced by environmental stress19 20 and by transfection of HSP genes.22–25

Abbreviations used in this paper: PHA, Phaseolus vulgaris lectin; PHA-E4, Phaseolus vulgaris isolecitin E4; WGA, wheat germ agglutinin; HSP, heat shock protein; BiP, binding protein; GlcNAc, N-acetylgalactosamine; LA, lactalbumin; DMEM, Dulbecco’s modified Eagle medium; FCS, fetal calf serum.
As a consequence of dietary intake, gut epithelial cells are regularly exposed to high levels of potentially harmful substances. Among these substances are the lectins which are contained in several foods of leguminous origin. At relatively high dietary intake lectins such as Phaseolus vulgaris lectin (PHA) and wheat germ agglutinin (WGA) can have harmful effects. Binding of lectins induces hyperplastic growth of the rat gut and pancreas, increased turnover of gut epithelial cells, polymannosylation of the mucosal membranes of the small intestine, and alterations of the activities of brush border enzymes. In vitro, apparently as a result of lectin binding to the cell membrane, changes are induced in the cellular metabolism of enterocyte-like fully differentiated Caco-2 cells. These effects in gut epithelial cells leading to severe disruptions in the integrity of the epithelial layer.

In the experiments presented in this study we focus on PHA and WGA. These substances have been described as potent growth factors for intestinal cells. To assess the physico-chemical stress caused by exposure to these lectins, we first screened slides made from the jejunum of rats which had been experimentally exposed to PHA or WGA for expression of HSPs in gut epithelium using monoclonal antibodies. Secondly, we used differentiated Caco-2 cells which display characteristics of small intestinal enterocytes both structurally and functionally to investigate if the lectins also induced changes in the heat shock response of these cells. In particular, we examined the effect of both lectins on expression of HSPs and established its extent.

Materials and methods

**LECTINS**

WGA with specificity for N-acetylglucosamine (GlcNAc) was isolated from wheat germ by affinity chromatography. Briefly, WGA soluble at pH 5.0 was heated at 60°C for 10 minutes and the supernatant fraction absorbed on to a column of immobilised GlcNAc. WGA was eluted from the column with 0.1 M GlcNAc. PHA with specificity for complex glycosyl side chains was isolated from red kidney beans by affinity chromatography on fetuin Sepharose-4B. WGA and Phaseolus vulgaris isolecithin E2 (PHA-E2), when used in cell culture experiments, were from E-Y Laboratories (San Mateo, California, USA).

**ANIMALS AND DIETS**

Six male, conventionally grown specific pathogen free inbred Hooded-Lister rats of the Rowett colony, kept singly in metabolism cages, were prefed for three days (6 g/rat/day) on a semisynthetic, good quality diet consisting mainly of maize starch, potato starch, and glucose. This diet contained 10% (w/w) lactalbumin (LA diet) as the sole protein. Rats weighing 80-90 g were divided into three groups of two rats each and pair-fed on different experimental diets for 10 days. The control group was kept on the same LA diet used for prefeeding. The WGA and PHA groups were fed diets based on the LA control diet in which 7% (w/w) of the lactalbumin was replaced by an equal amount of either WGA or PHA (93 g lactalbumin with 7 g WGA or PHA per kg diet). The food intake of all three groups was restricted to the voluntary intake of the PHA group (6 g diet/rat/day). This amount of diet contained 42 mg/day of WGA or PHA. On the morning of the 10th day, after overnight fasting, rats were offered 2 g of their respective diets and killed under ether anaesthesia two hours later. The abdomen was cut open and two gut sections of 2 cm each, 7 cm from the pylorus, were taken for histology and HSP immunostaining.

**TISSUE PROCESSING FOR HISTOLOGY AND HSP IMMUNOSTAINING**

Small intestinal segments, 2 cm in length, were fixed in 0.1 M phosphate buffered 4% formalin, pH 7.3, for 24 hours. After dehydration and embedding in paraffin, sections of 5 µm were cut.

After deparaffinization, sections were incubated with monoclonal antibodies against HSPs and subsequently processed with an avidin/biotin/peroxidase staining kit (Vectorstain, Vector Laboratories, Burlingame, California, USA). Endogenous peroxidase activity was blocked by exposure to methanol-hydrogen peroxidase for 15 minutes. In addition, the sections were counterstained with haematoxylin. The staining intensity of 20 well oriented crypts and villi per rat was estimated semiquantitatively. Estimations were performed at positions 10 villi apart from each other.

The specificity of the immunostaining was controlled by omitting the primary antibody, the secondary antibody, or the substrate. The specificity for the HSPs of the pattern showing lightly stained crypts and heavily stained villi was verified by incubating sections from lectin treated and control rat gut with an antiseraum directed against actin. These sections stained homogeneously in all cases.

Monoclonal antibodies against HSP60, HSP70, HSP72, BiP, and HSP90 were purchased from Stressgen Biotechnologies Corporation, Victoria, British Columbia, Canada (product numbers: SPA 800, SPA 806, SPA 810, SPA 822, and SPA 830, respectively) and processed according to the manufacturer’s instructions.

**CELL CULTURE**

The cell line Caco-2 was established from a moderately well differentiated colon adenocarcinoma. Caco-2 cells were routinely grown in Dulbecco’s modified Eagle’s medium (DMEM) (Flow Laboratories, Amsterdam, Netherlands). The medium was supplemented with 1% non-essential amino acids (Flow), 50 µg/ml gentamicin (Flow), 10 mM sodium bicarbonate, 25 mM HEPES, and 20% v/v fetal calf serum (FCS) (Sanbio, Uden, Netherlands) at 37°C with 5% CO2. Cells were seeded at 40 000 cells/cm2 in six well tissue culture plates (10 cm2) (Greiner, Alphen a/d Rijn, Nether-
lands). Cells were cultured for 19 days to achieve fully differentiated cell populations. Culture medium was changed three times a week.

For incubation with lectin, cells were incubated in medium without serum in order to be free from potentially reactive carbohydrates. Medium was first replaced with culture me-

Figure 1  Sections of the jejunum from rats fed diets containing lactalbumin (control), phytohaemagglutinin (PHA), or wheat germ agglutinin (WGA) stained with monoclonal antibodies to HSP70, HSP72, and HSP90. The jejunal sections of rats fed lactalbumin (A, D, G), PHA (B, E, H) or WGA (C, F, I) are shown. Sections A, B, and C were stained with the antibody to HSP70, sections D, E, and F with the antibody to HSP72, and sections G, H, and I with the antibody to HSP90.
was washed three times with 1 ml of 0.01 M PBS (0.01 M Na₂HPO₄, 0.01 M NaH₂PO₄, 0.9% (w/v) NaCl) pH 7.3, fixed with 1 ml of methanol for 10 minutes, and finally dissolved in 0.5 ml of 0.1 M NaOH. The incorporated radioactivity present in 0.2 ml of 0.1 M NaOH was determined by liquid scintillation counting in 2 ml of Dynagel (J T Baker Chemicals, Deventer, Netherlands).

Incorporation of [2-¹⁴C]thymidine and L-[methyl-³H]methionine by differentiated Caco-2 cells was also studied after exposure of the cells to 100 µg of heat inactivated WGA and PHA-E₄. Inactivation of the lectins was accomplished by heating the lectins for 60 minutes at 100°C.

Incorporated radioactivity, calculated as disintegrations per minute per µg DNA, was expressed as relative incorporation (factor by which incorporation was decreased or increased compared with cell cultures that were not incubated with PHA-E₄ or WGA).

Trypan blue exclusion was used to determine the effect of the lectins on cell viability. The cell monolayer was stained with 0.2% (w/v) trypan blue in 0.01 M PBS, pH 7.3, for five minutes. After decanting the dye cell counts of stained and dye excluding cells were made in situ.

**WESTERN BLOT ANALYSIS**

Cells from each well were solubilised in sample buffer containing 125 mM tris(hydroxymethyl) aminomethane HCl, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.0015% bromophenol blue, pH 6.8. Separation of proteins was achieved in about 30 seconds. DNA and protein synthesis in differentiated Caco-2 cells after exposure to lectins

**EXPOSURE TO LECTINS**

Exposure of the cells to WGA and PHA-E₄, was performed in quadruplicate in 0.5 ml of DMEM containing 100 µg/ml of WGA or PHA-E₄. After 7, 23, 31, 47, 55, and 79 hours, 100 µl of DMEM containing 0.05 µCi of [2-¹⁴C]thymidine (Amersham, Amersham, Netherlands) and 2 µCi of L-[methyl-³H]methionine (Amersham) were added to the monolayer and incubation was continued for another hour. Adding 1 ml of 10% (w/v) trichloroacetic acid (0°C) to the monolayer stopped incorporation of the radioactive precursors. Subsequently, the monolayer...
HSP CONTENT OF RAT GUT TISSUE

Gut tissue sections from rats fed lactalbumin (fig 1A, D, G) had a normal appearance. The villus-crypt ratio of the jejunum of these rats was about 4. There were striking changes in the morphology of the rat small intestine when rats were fed the lectin containing diet. The crypts of the jejunum of rats fed on diets containing PHA (fig 1B, E, H) or WGA (fig 1C, F, I) were elongated whereas the length of the villi was only slightly reduced. These gut tissue sections showed a villus-crypt ratio of 1–2. In addition, the hyperregenerative crypts displayed much higher mitotic activity compared with controls (data not shown).

Semiquantitative evaluation of the staining intensities is presented in table 1. The intensity of the anti-HSP immunostaining in rats fed the control diet, which represents the constitutive levels of HSPs, was equally strong in the whole section (fig 1A, D, G; table 1). In comparison with the gut sections stained with anti-HSP72 (fig 1D) and anti-HSP90 (fig 1G), the staining intensity of the control section with anti-HSP70 (fig 1A) was weaker.

Compared with the jejunal crypts of lactalbumin fed rats (fig 1A, D, G; table 1) the HSP content of the intestinal cells in the hyperregenerative crypts of PHA fed (fig 1B, E, H; table 1) and WGA fed (fig 1C, F, I; table 1) rats was decreased considerably. Also, the villi of these gut sections showed weaker intensity of anti-HSP immunostaining (fig 1A–F; table 1).

The strongest reduction in staining intensity was seen in the villi of rats fed PHA after immunostaining with anti-HSP72 (fig 1E) and anti-HSP90 (fig 1I), and also in rats fed WGA after immunostaining with anti-HSP90 (fig 1I). A decrease in the immunostaining intensity could not be found when rats were fed heat inactivated (60 minutes at 100°C) lectins (data not shown).

HSP CONTENT OF DIFFERENTIATED CACO-2 CELLS DURING LECTIN EXPOSURE

The lectins PHA-E, and WGA were used to determine the effect of lectin exposure on HSP synthesis in differentiated Caco-2 cells. The cellular content of HSP70 and HSP90 is given in fig 2, quantification of which and three other HSPs (HSP60, HSP72, and BiP) are given in fig 3. The results obtained from control Caco-2 cells clearly demonstrate that constitutive levels of HSPs are present in these enterocyte-like cells.

In general, the relative content of HSPs of non-proliferating differentiated Caco-2 cells decreased in time during exposure to 100 µg/ml of PHA-E, or WGA. The Western blot (fig 2) clearly demonstrated an inhibitory effect of PHA-E, on both HSP70 and HSP90. In contrast, WGA interfered only with the relative content of HSP70. This lectin has little or no effect on HSP90 content.

A striking difference was apparent between PHA-E, and WGA with respect to their effects on the relative content of HSPs.
on HSP synthesis (fig 3). With the exception of
HSP60, levels of all other proteins decreased in
time during exposure to PHA-E4. HSP60 was
different in that its cellular level remained
unchanged over time during lectin exposure. In
contrast, WGA caused an initial decrease in the
levels of HSPs during the first 24 hours of
exposure. This increase (except for HSP70)
was followed by partial (HSP60 and BiP) or
complete (HSP70 and HSP90) recovery of the
HSP content of the cells.

To determine if inhibition of HSP synthesis
by lectins was caused by an overall decrease in
cellular protein synthesis, differentia-
ted Caco-2 cells were pulse labelled for four hours
with methionine at various times after the start
of lectin exposure. During the first 24 hours of
exposure, relative incorporation did not
change. However, at selected points hereafter
both PHA-E4 and WGA appeared to stimulate
relative incorporation of methionine (fig 4).
From this experiment it is obvious that the cel-
lar protein synthesis machinery was not
inhibited. The incorporation curve for DNA
synthesis ran parallel to that of the protein.

Incubation of Caco-2 cells with 100 µg/ml of
PHA-E4 in the presence of 1 mg/ml of fetuin or
100 µg of WGA in the presence of 0.1 M
GlcNAc failed to induce the observed changes
in HSP synthesis (data not shown).

HSP RESPONSE IN LECTIN EXPOSED
DIFFERENTIATED CACO-2 CELLS
To investigate if the heat shock response in lec-
tin exposed differentiated Caco-2 cells was
impaired after lectin preincubation, these cells
were subsequently heat shocked for one hour
(40°C, 41°C, and 42°C) and allowed to recover
for six hours at 37°C. During heat shock and
the recovery period, lectin exposure was

Figure 6  HSP synthesis in differentiated Caco-2 cells after preincubation with lectins. Cells were exposed for 48 hours to
either 100 µg/ml of PHA-E4, or WGA and subsequently heat shocked for one hour at 37°C (control), 40°C, 41°C, or 42°C.
The cells were allowed to recover for six hours. Lectin exposure was continued for the duration of the heat shock and recovery
period. After these experimental procedures the cells were processed for western blotting and immunostaining as described.
Two different cell passages were used to establish the effect of PHA-E4, and WGA. For each passage the relative levels of
HSP60 (A), HSP70 (B), HSP72 (C), BiP (D), and HSP90 (E) were determined using triplicate cultures.
Stress response in gut epithelial cells

continued. HSP responses of HSP70 and HSP90 are given in the western blot in fig 5. Quantification of these and another three proteins (HSP60, HSP72, and BiP) is given in fig 6.

Heat shock did not induce all five HSPs. The heat shock response of control Caco-2 cells appeared to be restricted to HSP70, HSP72, and HSP90 (figs 5, 6B, C, E). At 42°C the strongest induction was achieved for these proteins. Heat shock caused little or no change in induction of HSP60 or BiP (fig 6A, D).

In PHA-E, exposed cells relative levels of HSP70, HSP72, and HSP90 decreased when cells received a heat shock at 40°C and 41°C. However, at 42°C significant induction of these proteins took place compared with the relative levels of control (37°C) and heat shocked cells (42°C). The effect on the relative levels of HSPs in WGA exposed cells was slightly different. HSP72 in particular was already induced at 40°C in WGA exposed cells. Another important observation is that in general, heat shock induced a stronger response in WGA exposed cells.

Western blots (fig 5) and their quantification (fig 6) clearly showed that differentiated Caco-2 cells which were preincubated with PHA-E, and WGA were still capable of synthesising HSPs after heat treatment at 42°C and to a lesser extent at 41°C. It is obvious from our results that the heat shock response in lectin preincubated differentiated Caco-2 cells was not impaired. Interestingly, enhancement of the heat shock response was observed for HSP70, HSP72, and HSP90.

Discussion

Cells have a highly conserved set of stress proteins (HSPs)1–9 which are involved in coping with chemical or physical damage. Our data clearly demonstrated that lectins, which are known to cause structural lesions in gut epithelial cells on exposure, interfere with the levels of HSPs in these cells. The HSP content in the crypt cells of the jejunum of PHA and WGA fed rats was dramatically decreased compared with the jejunum of lactalbumin fed rats (fig 1).

It could be argued that decreased levels of HSPs in these hyperregenerative crypts (fig 1B, C, E, F, H, I), which showed weaker intensity of anti-HSP60, HSP70, and HSP90 immunostaining, were caused by dilution of HSPs in the strongly dividing crypt cells. This observation was studied further in cultures of confluent non-proliferating differentiated Caco-2 cells.10 In general, the relative content of HSPs decreased during exposure to PHA-E, and WGA (figs 2, 3). WGA induced decreases recovered during the experiment. Only HSP60 levels remained unchanged during exposure to PHA-E. As there is little or no mitotic activity in the cultures used,11 dilution of HSPs into progeny cells cannot account for the decrease in HSP content. Therefore, this finding strongly suggests that increased mitotic activity was not responsible for the decrease in HSP levels.

The stress response is often associated with transient inhibition of the rate of total protein synthesis in cells.6–9 We investigated if alterations in the rate of protein synthesis in PHA-E, or WGA exposed cells could explain the observed depression of HSP levels (fig 4). Lectins do not interfere with protein synthesis during the first 24 hours of exposure. At later times the rate of protein synthesis in lectin exposed cells was even increased compared with control cells. These findings demonstrate that lower levels of HSPs in lectin exposed differentiated Caco-2 cells (figs 2, 3) do not result from an overall decrease in the rate of cellular protein synthesis.

The heat shock response in lectin exposed cells does not appear to be impaired by lectin incubation. Cells preincubated with PHA-E, or WGA were still capable of synthesising HSPs (figs 5, 6). When these cells were heat treated, levels of HSP70, HSP72, and HSP90 well exceeded the levels in heat shocked cells. Particularly at 42°C, the overshoot of HSP synthesis was conspicuous. This phenomenon resembles sensitisation which can occur during complex exposures to heat48 or sodium arsenite50 and may indicate the presence of sublethal damage to the cell.

The thymidine incorporation experiments suggest that prolonged incubation (24 hours or longer) with lectins stimulates DNA synthesis (fig 4). As cell viability remains relatively constant (>96%) and the DNA content per well does not change significantly during lectin exposure (data not shown), the change in thymidine incorporation most probably does not reflect increased DNA synthesis. Whether increased DNA repair occurs in these cells or whether the lectins interfere with nucleic acid metabolism in other ways is not known. Thymidine incorporation paralleled protein synthesis. To withstand tissue damage by noxious components in the gut lumen, intestinal cells have evolved protective mechanisms. HSPs can be expected to play a pivotal role in this protection.

To immediately cope with cell damage this tissue would benefit from high basal levels of HSPs. A high basal level of HSP72, the stress inducible form of HSP70, has been detected by western blot analysis in samples from rat colon.51 From the intensity of the anti-HSP immunostaining in the enterocytes of rats fed the lactalbumin diet it can be judged that high constitutive levels of HSP70, HSP72, and HSP90 are present in this tissue (fig 1A, D, G). By western blotting of samples from Caco-2 cells not exposed to lectins, we have revealed constitutive expression of HSPs (figs 2, 3, 5).

The mechanism by which HSP levels in rat gut cells and enterocyte-like Caco-2 cells are lowered by exposure to lectins is not apparent from our experiments. As synthesis of inducible HSPs following heat shock was not impaired by exposure to lectins (fig 6), levels of constitutive HSPs could be downregulated selectively. The speed of this downregulation suggests an active process. It is possible that...
HSPs are consumed in repairing sublethal cell damage without being adequately replenished. Lectins are known to interfere with the cytoskeleton of gut cells. Cytoskeletal lesions in rat enterocytes have been observed\(^ {27,31}\) and a shift in the ratio of globular to filamentous actin could be measured in differentiated Caco-2 cells.\(^ {37,37}\) This depolymerisation of the actin filaments takes place within a few minutes after the onset of lectin exposure. Studies on the function of HSP70\(^ {25}\) and HSP90\(^ {26}\) demonstrated actin binding activity by these proteins, which stabilises the actin filaments by cross linking. After lectin exposure, HSPs may be directed to the stabilisation of the cytoskeleton as well as the clathrin formation of cellular proteins. If the resulting demand for HSPs is not met by adequate synthesis, levels of soluble HSPs decrease.

It has been shown that binding of lectins to their specific sugar reactive sites on the apical membranes of enterocytes, being a prerequisite to exert its effect on the cell, is the first step in the damage caused by these dietary components. Whereas PHA-E, inhibits the heat shock response in gut epithelial cells (figs 1–3), PHA-L, is an activator of the stress response in lymphocytes,\(^ {25,57}\) acting by binding to lectin specific receptors on the cell membrane.

Considering the amount of evidence linking HSP levels to increased resistance to stress, it is likely that the lectins PHA-E, and WGA are detrimental not only to gut intestinal cells by damaging their apical membranes. Depression of the amounts of stress proteins in enterocytes by these lectins may leave the cells more vulnerable to exposure to agents produced by the digestive tract as well as to harmful agents of dietary origin. Considering that pathogenic bacteria such as Salmonella enteritidis and Escherichia coli induce stress proteins in intestinal cells,\(^ {38}\) lectin induced downregulation of these proteins may also indicate increased cellular susceptibility to bacterial invasion.

The Commission of European Communities supported this collaborative work. It was an European COST Action 98 programme coordinated by Dr A. Pusztai.