Physiologically relevant increase in temperature causes an increase in intestinal epithelial tight junction permeability

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Physiologically relevant increase in temperature causes an increase in intestinal epithelial tight junction permeability. Am J Physiol Gastrointest Liver Physiol 290: G204–G212, 2006; doi:10.1152/ajpgi.00401.2005.—The effects of physiologically relevant increase in temperature (37–41°C) on intestinal epithelial tight junction (TJ) barrier have not been previously studied. Additionally, the role of heat-shock proteins (HSPs) in the regulation of intestinal TJ barrier during heat stress remains unknown. Because heat-induced disturbance of intestinal TJ barrier could lead to endotoxemia and bacterial translocation during physiological thermal stress, the purpose of this study was to investigate the effects of modest, physiologically relevant increases in temperature (37–41°C) on intestinal epithelial TJ barrier and to examine the protective role of HSPs on intestinal TJ barrier. Filter-grown Caco-2 intestinal epithelial cells were used as an in vitro intestinal epithelial model system to assess the effects of heat exposure on intestinal TJ barrier. Exposure of filter-grown Caco-2 monolayers to modest increases in temperatures (37–41°C) resulted in a significant time- and temperature-dependent increases in Caco-2 TJ permeability. Exposure to modest heat (39 or 41°C) resulted in rapid and sustained increases in HSP expression; and inhibition of HSP expression produced a marked increase in heat-induced increase in Caco-2 TJ permeability (P < 0.001). Heat exposure (41°C) resulted in a compensatory increase in Caco-2 occludin protein expression and an increase in junctional localization. Inhibition of HSP expression prevented the compensatory upregulation of occludin protein expression and produced a marked disruption in junctional localization of occludin protein during heat stress. In conclusion, our findings demonstrate for the first time that a modest, physiologically relevant increase in temperature causes an increase in intestinal epithelial TJ permeability. Our data also show that HSPs play an important protective role in preventing the heat-induced disruption of intestinal TJ barrier and suggest that HSP mediated upregulation of occludin expression may be an important mechanism involved in the maintenance of intestinal epithelial TJ barrier function during heat stress.

Caco-2; heat-shock protein; tight junction protein

The intestinal epithelial barrier is composed of apical plasma membrane of the enterocytes (which form the transcellular barrier) and the intercellular tight junctions (TJ; which form the paracellular barrier) (18). Intact intestinal epithelial TJ barrier is crucial in providing barrier function against paracellular penetration of pathogenic bacteria and toxic luminal antigens including endotoxins (1, 33, 46, 50). The disruption of intestinal TJ barrier results in a “leaky” TJ barrier, allowing paracellular permeation of toxic luminal substances (2). It is well established that various types of stresses including hemorrhagic shock (2, 14, 15), endotoxemia (62, 66), psychogenic stress (54, 67, 68), exertional stress (3, 6), and heat stroke (20, 26, 30, 69) cause an increase in intestinal permeability to luminal endotoxins and lead to bacterial translocation (2, 3, 6, 14, 15, 20, 26, 30, 69). The gut-derived endotoxins and pathogenic bacteria have been proposed to be an important causative factor of morbidity and death during clinically relevant stresses such as heat stroke (22, 23), sepsis (72), burn injury (27, 71), ischemia-reperfusion injury (24), and in the critically ill (52).

Fever is a normal host response to infection (39). During normal febrile response, the core body temperature may range between 37 and 41°C (17, 49). Pediatric population often present with elevated temperatures of 39–41°C during acute infection (4, 9, 28, 39). Because prolonged exposure to core body temperature exceeding 40°C can result in serious complications including death, rapid cooling is warranted when core body temperature reaches 40°C (4, 28). The critical thermal maximum (CTM) is the minimal elevated core body temperature at which death occurs without rapid therapeutic intervention (36). The CTM for humans has been reported to be 41.6–42°C for 45 min to 8 h (4, 8). The elevation of core body temperature above the CTM of 41.6°C results in a rapid multiorgan failure and death (4). Previous studies have shown that elevation of core body temperature in rats to 42.5°C results in a rapid damage of intestinal epithelial surface with sloughing of the epithelial layer and cell death (43). The hyperthermia-induced damage of intestinal epithelial surface was also associated with a rapid increase in intestinal permeability (43, 69). In these studies, no morphological alternations in intestinal TJ complexes were seen (43). Despite the potential clinical significance, the effect of a physiologically relevant, modest increase in temperature on intestinal epithelial barrier has not been previously reported. Thus a major aim of this study was to determine whether physiologically relevant increase in temperatures from 37 to 41°C causes a disturbance in intestinal TJ barrier function.

It is well established that a wide variety of chemical (76) and physiological stresses (42, 45) causes a rapid increase in synthesis of heat-shock proteins (HSPs). The HSP expression is an important cellular response to heat stress and plays an important protective role against lethal injury (51, 57, 58). HSPs act as molecular chaperone binding to partially folded or
misfolded proteins and prevent their irreversible denaturation during heat stress (11, 44). The increased expression of HSP70 protects against lethal thermal injury (51, 57, 58), oxidative stress (58, 59), and endotoxin challenge (10, 35, 65). Despite the crucial importance of HSPs in protection against various types of lethal injury, the role of HSPs in maintaining or protecting intestinal epithelial TJ barrier during heat stress remains unknown. An important aim of this study was to also investigate the role of HSPs in protecting the intestinal TJ barrier during heat stress.

Heat stress has many biological effects in animals including inducing hypoperfusion of intestinal vasculature (41), activation of various inflammatory cascades and immune mediators (5), activation of apoptosis (38), activation of humoral system (40), and generation of oxygen free radicals (29) that are known to affect the intestinal TJ barrier. In this study, we used an in vitro intestinal epithelial system consisting of filter-grown Caco-2 intestinal epithelial monolayers to directly assess the effects of modest, physiologically relevant increases in temperature (37–41°C) on the intestinal epithelial TJ barrier in the absence of humoral, vascular, neuronal, or immunological factors. The filter-grown Caco-2 cells have been well validated as a useful in vitro epithelial model system to study intestinal epithelial barrier function and have been widely used for this purpose (32, 47, 73).

MATERIALS AND METHODS

Chemicals. Cell culture media (DMEM), trypsin, FBS, and related reagents were purchased from Life Technologies (Gaithersburg, MD). Glutamine, penicillin, streptomycin, and PBS were purchased from GIBCO-BRL (Grand Island, NY). Transwell permeable filters were purchased from Corning (Corning, NY). Anti-zonula occludens (ZO-1) antibody and anti-occludin antibody were obtained from Zymed Laboratories (San Francisco, CA). Quercetin, Triton X-100, bovine serum albumin, normal donkey serum, anti-β-actin antibody, and cycloheximide were purchased from Sigma (St. Louis, MO). Horseradish peroxidase (HRP)-conjugated secondary antibodies for Western blot analysis were purchased from Zymed Laboratories. Cy-5 antibodies for immunostaining were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Anti-HSP antibodies were purchased from Stressgen Biotechnologies (Victoria, British Columbia, Canada). Tween 20 and nonfat dry milk were purchased from Bio-Rad Laboratories (Hercules, CA). All other chemicals were of reagent grade and were purchased from Sigma, VWR (West Chester, PA), or Fisher Scientific (Pittsburgh, PA).

Cell cultures. Caco-2 cells (passage 18) were purchased from the American Type Culture Collection (Rockville, MD) and maintained at 37°C in a culture medium composed of DMEM with 4.5 mg/ml glucose, 50 μM penicillin, 50 μM streptomycin, 4 mM glutamine, and 25 mM HEPES and supplemented with heat-inactivated 10% FBS (32). Culture medium was changed every 2 days. After partial digestion with 0.25% trypsin and 0.9 mM EDTA in Ca²⁺ - and Mg²⁺-free PBS, Caco-2 cells were subcultured on Transwell filters for epithelial monolayer resistance and paracellular permeability studies. The filter-grown Caco-2 monolayers reached epithelial resistance of 400–550 Ω·cm² by 3–4 wk after seeding.

Determination of transepithelial resistance and paracellular permeability. An epithelial voltmeter (World Precision Instruments, Sarasota, FL) was used for measurements of the transepithelial electrical resistance (TER) of the filter-grown Caco-2 intestinal monolayers as previously reported (47). To study the time-course effects of heat stress on TER, Caco-2 monolayers were exposed to different temperatures ranging from 37 to 41°C over a 24-h time period. The effect of temperature on Caco-2 paracellular permeability was determined using an established paracellular marker inulin (48). For determination of mucosal-to-serosal flux rates of inulin, Caco-2-plated filters having epithelial resistance of 350–500 Ω·cm² were used. Known concentrations of permeability marker (1 μM) and its radioactive tracer were added to the apical solution. Low concentrations of permeability marker were used to ensure that negligible osmotic or concentration gradient was introduced.

Assessment of HSP, occludin, ZO-1, and β-actin protein expression by Western blot analysis. To study the time-course effect of heat stress on HSP and TJ protein expression, Caco-2 monolayers were exposed to elevated temperatures for varying time periods. At the end of the experimental period, Caco-2 monolayers were immediately rinsed with ice-cold PBS, and cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 500 μM NaF, 2 mM EDTA, 100 μM vanadate, 100 μM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 40 mM paranitrophenyl phosphate, 1 μg/ml aprotinin, and 1% Triton X-100) and scraped, and the cell lysates were placed in Microfuge tubes. Cell lysates were centrifuged to yield a clear lysate. Supernatant was collected, and protein measurement was performed using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories). Laemmli gel loading buffer was added to the lysate containing 5–10 μg of protein and boiled for 5 min, after which proteins were separated on an SDS-PAGE gel. Proteins from the gel were transferred to the membrane (Trans-Blot Transfer Medium, Nitrocellulose Membrane; Bio-Rad Laboratories) overnight. The membrane was incubated for 2 h in blocking solution (5% dry milk in TBS-Tween 20 buffer). The membrane was incubated with appropriate primary antibodies in blocking solution. After being washed in TBS-Tween buffer, the membrane was incubated in appropriate secondary antibodies and developed using the Santa Cruz Western Blotting Luminol Reagents (Santa Cruz Biotechnology, Santa Cruz, CA) on the Kodak BioMax MS film (Fisher Scientific, Pittsburgh, PA).

Immunostaining of TJ proteins. For immunostaining of TJ proteins, Caco-2 monolayers grown on coverslips were exposed to appropriate experimental conditions. At the end of the experimental period, Caco-2 monolayers were washed twice in cold PBS and were fixed with 2% paraformaldehyde for 20 min. Then cells were permeabilized with 0.1% Triton X-100 in PBS at room temperature for 20 min. The Caco-2 monolayers were then incubated in blocking solution composed of bovine serum albumin and normal donkey serum in PBS for 1 h. Cells were then labeled with primary antibodies in blocking solution overnight at 4°C. After being washed with PBS, the coverslips were incubated in Cy-5-conjugated secondary antibody for 1 h at room temperature and mounted on microscope slides (Erie Scientific, Portsmouth, NH). Immunolocalizations of TJ proteins were visualized using a Nikon fluorescence microscope (Nikon, Garden City, NY) equipped with a Hamamatsu digital camera (Hamamatsu Photonics, Hamamatsu, Japan). Images were processed with Wasabi software (Hamamatsu Photonics Deutschland, Herrsching, Germany).

Assessment of apoptosis and cell death. Caco-2 apoptosis was assessed by annexin V-FITC labeling (7, 12, 13, 16, 74). After the appropriate experimental treatment, Caco-2 cells were trypsinized and assessed for apoptosis using the annexin V-FITC apoptosis detection kit II from BD Sciences Pharmingen (San Diego, CA). Annexin V-FITC was used to stain for the apoptotic cells, and propidium iodide (PI) was used to stain the necrotic cells (12, 13, 74). PI is a fluorescent vital dye that stains DNA. In live cells, PI does not cross the intact plasma membrane of cells. In necrotic or dead cells, plasma membrane becomes permeable to PI for staining of DNA. Apoptosis or necrosis was measured by flow cytometry using a FACScan flow cytometer (BD Biosciences, San Jose, CA). Fluorescence of all dyes was excited with the 488-nm line of an argon ion laser. Fluorescence emission was detected in the FL-1 channel (530 ± 15 nm) for cells labeled with annexin V-FITC and in the FL-2 channel (585 ± 21 nm) for cells labeled with PI; subsequently, an FL-1/FL-2 dot plot was generated. For each experimental sample, a total of 20,000 cells were counted for annexin V-FITC and PI stain. For lactose dehydrogenase.
(LDH) measurements, the cellular medium was collected, and the enzyme activity was monitored spectrophotometrically using a Promega assay kit (Promega, Madison, WI).

**Statistical analysis.** Results are expressed as means ± SE. Statistical significance of differences between mean values was assessed with Student’s t-tests for unpaired data. All reported significance levels represent two-tailed P values. A P value of <0.05 was used to indicate statistical significance. All experiments were repeated a minimum of three times to ensure reproducibility.

**RESULTS**

**Effect of physiologically relevant increase in temperature (37–41°C) on Caco-2 intestinal epithelial TJ barrier.** The effect of a modest, physiologically relevant increase in temperature on Caco-2 transepithelial resistance (TER) and paracellular permeability was examined over a 24-h experimental period. Increasing temperatures from 37 to 41°C produced a significant time- and temperature-dependent drop in TER (P < 0.001; Fig. 1A). The effect of heat exposure on Caco-2 paracellular permeability was also examined by measuring transepithelial flux of paracellular marker inulin. Increasing temperature to 41°C resulted in a time-dependent increase in mucosal-to-serosal flux of inulin (P < 0.001; Fig. 1B). Plot of heat effect on TER and paracellular permeability revealed a linear inverse relationship (Fig. 1C), indicating that the heat-induced drop in TER correlates directly with the increase in paracellular permeability (r = 0.96).

To determine whether heat stress-induced decrease in TER is reversible after 24-h heat exposure (41°C), Caco-2 monolayers were returned to control temperature of 37°C. The reexposure to the normal control temperature of 37°C resulted in a rapid increase in Caco-2 TER to baseline levels, indicating that the drop in Caco-2 TER was rapidly reversible (Fig. 2).

**Effect of modest heat exposure on inducible and constitutively active HSP70 protein expression.** In the following studies, the effect of modest heat exposure (39 or 41°C) on expression of inducible and constitutively active forms of HSP70 in Caco-2 cells was determined. By 2 h of heat exposure, there was a modest increase in inducible HSP70 protein expression (Fig. 3). The increase in HSP70 protein expression reached maximal levels by 4–8 h of heat exposure and remained elevated throughout the 24-h experimental period. In contrast, the level of constitutively expressed HSP (HSC70) remained unchanged throughout the 24-h heat-exposure period (Fig. 3). The exposure of Caco-2 cells to a temperature of 39°C also caused a significant increase in HSP70 expression but to a slightly lesser extent than at 41°C (data not shown). Next, the effect of modest heat exposure on expression of other HSPs including HSP27, HSP40, and HSP90 was examined. Increasing temperature to 41°C also resulted in a significant increase in the expression of HSP27, HSP40, and HSP90 in Caco-2 cells without affecting the protein expression of internal control β-actin (Fig. 4), indicating that modest heat exposure induces a generalized increase in HSP expression.

**Effect of HSP inhibitor quercetin on modest heat-induced increase in HSP70 expression and Caco-2 TJ barrier.** In the following studies, the possible protective role of HSP expression on heat-induced disturbance in Caco-2 TJ barrier was examined. In these studies, we utilized a commonly used HSP inhibitor quercetin (which inhibits heat shock factor activation) to inhibit the heat stress-induced increase in HSP expression (34, 60). Quercetin (100 μM) treatment, at a dose previously shown to inhibit the HSP expression in Caco-2 cells, inhibited the heat (41°C) induced increase in HSP70 protein expression (Fig. 5) (63). (Quercetin was added to the incubation media 30 min prior to heat exposure and was left in place for the entire 24-h experimental period). The expression of constitutively
The exposure to high temperatures (37°C-41°C) is known to induce cell apoptosis and necrosis (31, 38). The possibility that the modest heat (37°C-41°C) induced increase in Caco-2 TJ permeability may also be related to cell apoptosis or necrosis was examined. The effect of heat stress on Caco-2 apoptosis and necrosis was examined by annexin V-FITC labeling. In the early stages of apoptosis, phosphatidylserine located on the inner (cytoplasmic) leaflet of the plasma membrane in healthy cells translocates to the outer leaflet (or external surface) of the membrane. The translocation of phosphatidylserine to the external surface of the plasma membrane allows annexin V-FITC to bind directly to the cells undergoing apoptosis. Thus cells undergoing apoptosis stain positive for annexin V-FITC. In cells undergoing necrosis, propidium iodide is able to cross the leaky plasma membrane and stain the DNA of cells undergoing necrosis. The exposure of Caco-2 cells to modest heat (41°C) for the 24-h experimental period did not have any significant effect on annexin V-FITC labeling or propium iodide staining, indicating that heat exposure up to 41°C does not induce apoptosis or necrosis in Caco-2 cells (Table 1). Moreover, quercetin treatment during modest heat (41°C) also did not induce apoptosis or necrosis. The cytotoxic effect of heat stress and quercetin treatment on Caco-2 cells was also confirmed by LDH release studies (Table 2). The exposure of Caco-2 monolayers to heat (41°C) in the presence or absence of quercetin (100 μM) did not have any significant effect on LDH release (Table 2). In addition, microscopic evaluation of the Caco-2 monolayers throughout the heat exposure revealed intact monolayers without denudation of monolayers, floating of dead cells, or formation of large gaps between cells. Together, these findings expressed heat shock protein was unaffected by the quercetin treatment (Fig. 5). Next, the effect of HSP inhibition on heat (41°C) induced increase in Caco-2 TJ permeability was examined. At control temperature of 37°C, quercetin (100 μM) did not affect the Caco-2 TER or paracellular permeability. In contrast, quercetin treatment at 41°C resulted in a significantly \( (P < 0.001) \) greater increase in Caco-2 TJ permeability as indicated by the marked drop in Caco-2 TER and increase in paracellular permeability (Fig. 6). These findings suggested that the heat-induced HSP protein expression had a protective effect on the heat-induced disturbance in Caco-2 TJ barrier.

**Effect of protein synthesis inhibitor cycloheximide on heat-induced increase in HSP70 expression and Caco-2 TJ permeability.** To further validate the role of heat-induced HSP expression in protecting the Caco-2 TJ barrier during heat stress, HSP expression was inhibited by cycloheximide (a protein synthesis inhibitor). Cycloheximide treatment (3 μg/ml) inhibited the heat-induced increase in HSP70 protein expression (Fig. 7A). Additionally, cycloheximide inhibition of HSP70 expression resulted in a significantly greater drop in Caco-2 TER following heat exposure \( (P < 0.001) \) (Fig. 7B), further substantiating the role of HSP expression in preventing the heat-induced increase in Caco-2 TJ permeability.

**Role of apoptosis or cell death in heat-induced increase in Caco-2 TJ permeability.** Since exposure to high temperatures (41.5°C-45°C) is known to induce cell apoptosis and necrosis (31, 38), the possibility that the modest heat (37°C-41°C) induced increase in Caco-2 TJ permeability may also be related to cell apoptosis or necrosis was examined. The effect of heat stress on Caco-2 apoptosis and necrosis was examined by annexin V-FITC labeling. In the early stages of apoptosis, phosphatidylserine located on the inner (cytoplasmic) leaflet of the plasma membrane in healthy cells translocates to the outer leaflet (or external surface) of the membrane. The translocation of phosphatidylserine to the external surface of the plasma membrane allows annexin V-FITC to bind directly to the cells undergoing apoptosis. Thus cells undergoing apoptosis stain positive for annexin V-FITC. In cells undergoing necrosis, propidium iodide is able to cross the leaky plasma membrane and stain the DNA of cells undergoing necrosis. The exposure of Caco-2 cells to modest heat (41°C) for the 24-h experimental period did not have any significant effect on annexin V-FITC labeling or propium iodide staining, indicating that heat exposure up to 41°C does not induce apoptosis or necrosis in Caco-2 cells (Table 1). Moreover, quercetin treatment during modest heat (41°C) also did not induce apoptosis or necrosis. The cytotoxic effect of heat stress and quercetin treatment on Caco-2 cells was also confirmed by LDH release studies (Table 2). The exposure of Caco-2 monolayers to heat (41°C) in the presence or absence of quercetin (100 μM) did not have any significant effect on LDH release (Table 2). In addition, microscopic evaluation of the Caco-2 monolayers throughout the heat exposure revealed intact monolayers without denudation of monolayers, floating of dead cells, or formation of large gaps between cells. Together, these findings...
indicated that modest heat exposure up to 41°C did not induce cell apoptosis or necrosis or denudation of epithelial layer, excluding cell death and cell sloughing as a mechanism for the increase in Caco-2 TJ permeability.

Effect of modest heat exposure on expression of Caco-2 tight junction proteins occludin and ZO-1. In the following studies, the effect of modest increase in temperature on expression of TJ proteins occludin and ZO-1 was examined by Western blot analysis (Fig. 8). The exposure of Caco-2 monolayers to 41°C temperature resulted in a progressive decrease in ZO-1 protein expression. In contrast, exposure to 41°C resulted in a progressive increase in occludin protein expression (Fig. 8). Exposure to 39°C also caused an increase in occludin expression but to a lesser extent (data not shown). Since above studies indicated that HSP expression was important in protection against the heat-induced increase in Caco-2 TJ permeability, in the following studies we examined the possibility that the heat-induced increase in Caco-2 TJ permeability, in the following studies we examined the possibility that the heat-induced increase in Caco-2 TJ permeability was mediated by HSP expression. The quercetin inhibition of HSP expression completely inhibited the heat-induced increase in occludin protein expression (Fig. 9), suggesting that heat-induced HSP expression was also required for the up-regulation of occludin protein expression. In contrast, quercetin pretreatment did not affect heat-induced decrease in ZO-1 protein expression (Fig. 9).

Effect of modest heat exposure on junctional localization of occludin. The effect of heat exposure on junctional localization of occludin was determined by immunostaining (Fig. 10). In the control Caco-2 monolayers, occludin proteins were localized at the cellular junctions and appeared as a continuous band encircling the cells at the apical cellular borders (Fig. 10A). Heat (41°C) or quercetin treatment alone did not affect the occludin localization (Fig. 10, B and C). However, it should be noted that the heat exposure caused a qualitative increase in the intensity of occludin staining (Fig. 10C), consistent with the increase in protein expression (Fig. 8). The quercetin treatment during heat exposure (41°C) caused a marked disruption in junctional localization of occludin protein with a visible discontinuity and gaps in occludin localization and a decrease in the overall intensity of occludin staining (Fig. 10D). These changes in occludin staining were corroborated by a significant decrease in the relative resistance of the Caco-2 monolayers (Fig. 6A).

**Table 1. The effect of moderate heat stress and quercetin on Caco-2 cell apoptosis and necrosis**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>%Cells Undergoing Apoptosis</th>
<th>%Cells Undergoing Necrosis</th>
</tr>
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<tbody>
<tr>
<td>37°C</td>
<td>5.34±0.96</td>
<td>2.74±1.11</td>
</tr>
<tr>
<td>41°C</td>
<td>6.45±0.52 (0.36)</td>
<td>3.19±0.36 (0.72)</td>
</tr>
<tr>
<td>41°C + Q</td>
<td>5.66±0.57 (0.79)</td>
<td>4.47±0.25 (0.21)</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 3. The effect of heat stress and quercetin on Caco-2 cell apoptosis was assessed by annexin V-FITC labeling, and cell necrosis was assessed by propidium iodide as described in MATERIALS AND METHODS. Caco-2 monolayers were exposed to heat (41°C) alone or with quercetin (Q) (100 µM) for a 24-h period. Caco-2 apoptosis (positive stain for annexin V-FITC) or necrosis (positive stain for propidium iodide) was detected by using a FACScan flow cytometer. P value in parentheses vs. 37°C.
findings suggested a structural-functional correlation between disruption of junctional localization of occludin protein and disturbance in Caco-2 TJ barrier function.

**DISCUSSION**

In this study, we have shown for the first time that a modest, physiologically relevant increase in temperature from 37° to 41°C causes a significant temperature-dependent disturbance in intestinal epithelial TJ barrier. Our results also suggested for the first time that HSP expression plays an important protective role in preventing the heat-induced disruption of the intestinal TJ barrier. Moreover, our results also indicated that HSP protection of intestinal epithelial TJ barrier during heat stress correlated with an up-regulation and maintenance of junctional localization of transmembrane TJ protein occludin.

Previous studies have shown that exposure to temperatures equal to or above the critical thermal maximum (CTM) of 41.6°–42.0°C results in a rapid increase in intestinal epithelial permeability (43). As previously defined CTM is a core body temperature at or above which a lethal injury occurs (4, 8). The mean blood levels of lipopolysaccharide (LPS) in heat stroke patients was more than 1,000-fold higher than in control subjects (8.6 ± 1.19 ng/ml in heat stroke patients vs. < 9 pg/ml in controls) (5). The clinical importance of intestinal epithelial permeation of gut endotoxins in heat stroke related deaths has been supported by studies in primates showing that antibiotic treatment prior to heat exposure prevented the heat-induced endotoxemia (25), and that anti-lipopolysaccharide antibody have found that primates exposed to temperatures of 41.5° to 45.0°C had a significant leakage of gut endotoxin into portal vein (20–23, 26). Similarly, Bouchama et al.(5) found that in humans diagnosed with heat stroke, all 17 heat stroke patients had markedly elevated blood levels of gut-derived endotoxin.

Table 2. The effect of moderate heat stress and quercetin on Caco-2 cell death as determined by LDH release

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Relative LDH Release</th>
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<tbody>
<tr>
<td>37°C</td>
<td>1.00 ± 0.20</td>
</tr>
<tr>
<td>41°C</td>
<td>0.80 ± 0.07 (0.23)</td>
</tr>
<tr>
<td>41°C + Q</td>
<td>0.82 ± 0.09 (0.29)</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 3. The effect of heat stress and Q on Caco-2 cell death was assessed by lactose dehydrogenase (LDH) release. Caco-2 monolayers were exposed to heat (41°C) alone or with Q (100 μM) over a 24-h period. Cell death was determined by LDH release as detected by colorimetric measurement.

Fig. 9. The effect of HSP inhibitor quercetin on heat stress-induced changes in occludin and ZO-1 protein expression. Caco-2 monolayers were exposed to 41°C for the 24 h in the presence or absence of quercetin (Q) (100 μM). Subsequently, occludin and ZO-1 protein expression was determined by Western blot analysis as described in MATERIALS AND METHODS. Treatment with quercetin at 41°C inhibited the heat stress-induced increase in occludin protein expression and did not affect heat-induced decrease in ZO-1 protein expression. β-actin served as an internal control.

**Table 2. The effect of moderate heat stress and quercetin on Caco-2 cell death as determined by LDH release**

Fig. 8. Time-course effect of heat exposure (41°C) on tight junction protein expression in Caco-2 monolayers. Caco-2 monolayers were exposed to 41°C for increasing time points (0–24 h). Occludin and ZO-1 protein expressions were determined by Western blot analysis as described in MATERIALS AND METHODS. Heat exposure resulted in a progressive increase in occludin protein expression and a decrease in ZO-1 protein expression over the 24-h treatment period. β-actin served as an internal control.

**Fig. 9.** The effect of heat stress and quercetin on tight junction protein expression in Caco-2 monolayers. Caco-2 monolayers were exposed to 41°C for the 24 h in the presence or absence of quercetin (Q) (100 μM). Subsequently, occludin and ZO-1 protein expression was determined by Western blot analysis as described in MATERIALS AND METHODS. Treatment with quercetin at 41°C inhibited the heat stress-induced increase in occludin protein expression and did not affect heat-induced decrease in ZO-1 protein expression. β-actin served as an internal control.

**Fig. 8.** Time-course effect of heat exposure (41°C) on tight junction protein expression in Caco-2 monolayers. Caco-2 monolayers were exposed to 41°C for increasing time points (0–24 h). Occludin and ZO-1 protein expressions were determined by Western blot analysis as described in MATERIALS AND METHODS. Heat exposure resulted in a progressive increase in occludin protein expression and a decrease in ZO-1 protein expression over the 24-h treatment period. β-actin served as an internal control.

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prettreatment prior to heat exposure also prevented the fatality related to heat stroke (22). The mortality of primates during heat-stroke was directly related to the presence of endotoxemia (20–23, 26). In sum, these studies suggested that exposure to temperatures above the CTM of 41.6°–42.0°C leads to a rapid damage of intestinal epithelial layer and an increase in intestinal permeability to toxic luminal antigens including endotoxins culminating in endotoxemia, which directly contributes to multi-organ dysfunction and death related to heat stroke (4).

While it is well-established that exposure to temperatures exceeding the CTM of 41.6°–42.0°C induces a rapid sloughing of intestinal epithelial surface and an increase in intestinal permeability (43), the effects of more modest, physiologically relevant increases in temperature (37°–41°C) on intestinal epithelial barrier function had not been previously described. In the present study, we show for the first time that a modest increase in temperatures from 37° to 41°C causes a significant temperature dependent increase in intestinal epithelial TJ permeability (1). In contrast to the previous studies showing rapid intestinal epithelial cell death and denudation of epithelial layer during exposure to temperatures (42°–45°C) exceeding the CTM, our present data indicated that a modest increase in temperatures between 37°–41°C does not induce epithelial cell apoptosis or necrosis or denudation of epithelial monolayer (Table 1 and 2). Our data indicated that morphological appearance of intestinal monolayers remained intact throughout the heat exposure without an increase in LDH release. Moreover, the heat-induced drop in Caco-2 TER directly correlated with an increase in flux rates of paracellular markerulin (Fig. 1C). In combination, our data indicated that the increase in Caco-2 epithelial permeability was not due to heat-induced apoptosis or cell death, but a selective increase in Caco-2 TJ permeability.

Heat shock proteins play a crucial role in protecting against lethal injury from various types of stresses (57, 59, 65). Previous in-vitro cell culture and in-vivo animal studies have shown that heat-induced HSP70 expression protects against different types of lethal injury. For example, induction of HSP70 expression by brief preconditioning heat treatment (43°C for 30 min) prior to heat stress prevented the heat stress (43°C for 90 min) induced apoptosis in human T lymphocytes in a HSP70 dependent manner (57). The HSP70 induction in various cell types also prevented the stress-induced apoptosis by ceramide treatment and ATP depletion (56, 64). Additionally, heat stress induced HSP70 or HSP72 expression prior to endotoxin infusion resulted in protection against endotoxin induced fatality in rats (10, 65). It has also been shown that preconditioning heat treatment in MDCK cells produced a physiological thermotolerance (55). The importance of HSP inhibition in inducing cell death was also demonstrated by studies showing that HSP70 inhibition by quercetin produced a marked enhancement of heat-induced cytotoxicity (37, 61).

Despite the crucial importance of HSPs in protection against various types of lethal injury, the role of HSPs in maintaining or protecting the intestinal epithelial TJ barrier during heat stress has not been previously reported. In this study, we show for the first time that heat-induced HSP expression played an important role in the maintenance of the Caco-2 TJ barrier function during a physiologically relevant increase in temperature. Our data indicated that in response to modest heat exposure (37°–41°C), Caco-2 cells rapidly produced HSPs (Figs. 3 and 4); and inhibition of HSP expression by quercetin or cycloheximide resulted in a marked increase in Caco-2 TJ permeability during heat stress (41°C) (Figs. 6 and 7). Thus our findings suggested that HSPs have a direct protective role in maintaining the intestinal epithelial TJ barrier during physiologically relevant heat stress. Since endotoxemia has been proposed as an important pathogenic factor involved in the hyperthermia-related deaths (5, 22, 23), the HSP-mediated protection of intestinal TJ barrier could play an important role in attenuating the extent of endotoxemia during heat stress. Therefore, therapeutic strategies which enhance or maintain HSP expression could be important in preserving the intestinal TJ barrier function during heat stress and preventing the development of endotoxemia.

The effect of heat stress on intestinal epithelial TJ protein expression has not been previously reported. Previous studies in rats indicated that acute exposure to elevated temperatures of 41.5°–43.0°C did not affect the appearance of intestinal epithelial TJs as visualized by transmission electron microscopy (43). As the rat intestinal epithelial layer was denuded shortly after core body temperature was raised to 41.5°C, longer effects of elevated core temperature on intestinal TJ complex were not examined (43). In the present study, our data indicated that a modest increase in temperatures to 41°C resulted in a progressive decrease in expression of Caco-2 ZO-1 protein that was associated with heat stress-induced increase in Caco-2 TJ permeability. The functional significance of decrease in ZO-1 protein expression during heat stress is unclear. One possibility is that the decrease in expression may be a contributing factor leading to the disturbance in the TJ barrier function. In contrast, there was a compensatory increase in occludin protein expression during heat exposure (Fig. 8). Since previous studies have shown a direct correlation between increase in occludin protein expression and increase in TJ barrier function (19, 53, 77), we also propose that the heat-induced increase in Caco-2 occludin protein expression could also have a TJ barrier enhancement effect during physiological heat stress (Fig. 8). The potential importance of occludin expression in the formation and enhancement of TJ barrier function has been well-demonstrated by studies showing that expression of occludin in fibroblasts results in an increased cell-to-cell adhesion in transfected fibroblasts, while peptide inhibitor of occludin prevents the adhesion (75). Increased expression of occludin in MDCK cells by gene transfection also produced a significant increase in TER and an increase in the number of TJ strands (53). Moreover, numerous studies have shown a direct correlation between a decrease in intestinal epithelial TJ barrier function and a decrease in occludin expression or dissociation of occludin from the TJ complex (70).

Our results also suggested that the heat-induced HSP expression was required for the up-regulation of occludin protein expression. The inhibition of HSP expression prevented the up-regulation of occludin expression in Caco-2 monolayers (Fig. 9) and caused a marked disruption in the junctional localization of occludin protein during heat exposure (Fig. 10). Thus inhibition of occludin protein expression and disturbance in junctional localization of occludin directly corresponded to increase in Caco-2 TJ permeability (Fig. 6). Although the precise mechanisms involved in HSP-mediated increase in occludin protein expression remain to be elucidated, there are several possibilities. One possibility is that HSPs may have a...
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direct influence on occludin protein expression by functioning as molecular chaperone binding to partially folded or misfolded occludin proteins and preventing the heat-induced aggregation and denaturation of occludin protein. Second possibility is that HSPs stabilize occludin mRNA, leading to an increase in occludin protein synthesis. Another possibility is that HSPs may have a direct effect on regulation of occludin promoter activity resulting in an increase in gene transcription. Recently, we have initiated studies to address the possible intracellular mechanisms involved in the HSP up-regulation of occludin proteins. In support of the chaperone function for HSP70 in stabilizing the occludin protein during heat stress, our preliminary studies have shown that heat exposure (41°C) causes an increase in binding of HSP70 to occludin protein (unpublished data). At control temperature of 37°C, there was no co-immunoprecipitation of HSP70 with occludin. In contrast, increasing temperature to 41°C resulted in a marked increase in HSP70 co-immunoprecipitation with occludin, indicating increased binding of HSP70 to occludin protein during heat stress. In future studies, we intend to further examine the chaperone function of HSP70 and also examine the role of HSPs on the stability of occludin mRNA and occludin promoter activity.

In conclusion, our results indicate that exposure to physiologically relevant increase in temperatures from 37° to 41°C produces a modest but significant increase in Caco-2 TJ permeability. The heat-induced HSP expression appeared to play a central protective role in preventing the heat-induced disturbance in Caco-2 TJ barrier. The HSP-mediated protection of Caco-2 TJ barrier during heat stress was closely associated with up-regulation of occludin protein expression and junctional localization of occludin proteins. Thus our data suggested for the first time that the heat stress-induced HSP expression plays an important protective role in maintaining the intestinal epithelial TJ barrier function during physiologically relevant heat stress.

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