Polyamines mediate glutamine-dependent induction of the intestinal epithelial heat shock response

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Iwashita Y, Sakiyama T, Musch MW, Ropeleski MJ, Tsubouchi H, Chang EB. Polyamines mediate glutamine-dependent induction of the intestinal epithelial heat shock response. Am J Physiol Gastrointest Liver Physiol 301: G181–G187, 2011. First published April 21, 2011; doi:10.1152/ajpgi.00054.2011.—Heat shock proteins (Hsps) are highly conserved proteins that play a role in cytoprotection and maintaining intestinal homeostasis. Glutamine is essential for the optimal induction of intestinal epithelial Hsp expression, but its mechanisms of action are incompletely understood. Glutamine is a substrate for polyamine synthesis and stimulates the activity of ornithine decarboxylase (ODC), a key enzyme for polyamine synthesis, in intestinal epithelial cells. Thus we investigated whether polyamines (putrescine, spermidine, or spermine) and their precursor ornithine mediate the induction of Hsp expression in IEC-18 rat intestinal epithelial cells. As previously observed, glutamine was required for heat stress induction of Hsp70 and Hsp25, although it had little effect under basal conditions. Under conditions of glutamine depletion, supplementation of ornithine or polyamines restored the heat-induced expression of Hsp70 and Hsp25. When ODC was inhibited by α-difluoromethylornithine (DFMO), an irreversible ODC inhibitor, the heat stress induction of Hsp70 and Hsp25 was decreased significantly, even in the presence of glutamine. Ornithine, polyamines, and DFMO did not modify the nuclear localization of heat shock transcription factor 1 (HSF-1). However, DFMO dramatically reduced glutamine-dependent HSF-1 binding to an oligonucleotide with heat shock elements (HSE), which was increased by glutamine. In addition, exogenous polyamines recovered the DNA-binding activity. These results indicate that polyamines play a critical role in the glutamine-dependent induction of the intestinal epithelial heat shock response through facilitation of HSF-1 binding to HSE.

glutamine; heat shock protein; polyamine; ornithine; heat shock transcription factor 1

GLUTAMINE IS THE MOST ABUNDANT amino acid in the blood (38). Although considered nonessential, glutamine becomes conditionally essential under conditions of critical illness, postsurgical stress, chronic inflammation, or malnutrition in which glutamine is depleted rapidly from the body (1, 29). Recent studies have demonstrated that supplementation of parenteral or enteral glutamine reduced complication rates in critically ill and postoperative patients (9, 10, 26). Because intestinal epithelial cells use glutamine as their principal metabolic fuel, the gastrointestinal tract is particularly susceptible to injury under stress conditions, which can cause increased intestinal permeability and bacterial translocation. In the intestine, glutamine plays a protective role during physiological stress, since it is required for the induction of heat shock proteins (Hsps) (6, 42). Glutamine also regulates intestinal epithelial cell proliferation (3, 16) and autophagy (34).

Hsps are a highly conserved family of molecular chaperones from prokaryotes to humans (7, 19). Inducible Hsps are produced in response to stressors, including hypoxia, oxidative radicals, hyper- and hypothermia, heavy metals, and malnutrition. Hsps have a pivotal role in protecting intestinal epithelial cells from transport and barrier changes associated with gut inflammation (13, 27). Inducible Hsps, in particular Hsp70 and Hsp25, are constitutively expressed by the surface epithelial cells of the stomach and colon in response to the inherent acidic pH (36) and bacterial-inducing signals (17), respectively. Glutamine enhances the expression of Hsp70 and Hsp25 in intestinal epithelial cells, an effect that confers protection against oxidant injury (39, 42, 43). The regulation of Hsp expression is mediated by heat shock transcription factor 1 (HSF-1), which is activated upon heat stress through a cascade of posttranslational modifications, including trimerization, translocation to the nucleus, binding to heat shock elements (HSE), and the phosphorylation-dependent transcriptional activation at HSE (25, 41, 44). We have shown that glutamine is essential for the optimal induction of Hsp70 in intestinal epithelial cells (33). In its absence, the heat shock response is markedly attenuated during conditions of stress. Glutamine specifically augments heat-induced Hsp70 transcript abundance, however, no glutamine-dependent differences in HSF-1 phosphorylation, trimerization, or nuclear localization were found during heat shock in intestinal epithelial cells. Although glutamine potentiation of the Hsp70 response is thought to be mediated through modulation of the binding of HSF-1 to HSE, it is still unclear what key molecules are involved in the DNA binding. We previously demonstrated that a significant portion of glutamine’s actions for heat induction of Hsp25 is the result of its metabolism to glutamate (31). The effect of glutamate was less than glutamine, which could be accounted for by decreased glutamate uptake. However, increasing medium glutamate concentration and longer incubation times were required for glutamate to have this effect, which at a maximum was <50% of the maximal effect of glutamine (31).

Once in the cell, glutamate can be used by intestinal epithelial cells to produce other amino acids, including aspartate, alanine, praline, and ornithine. Neither aspartate, alanine, nor proline supported the heat induction of Hsp25 (31). Arginine, which can also be used for polyamine synthesis, has, similar to glutamate, much lower uptake than glutamine. However, we did not determine (as for glutamate) whether increasing medium concentration or duration of exposure would enhance the effects of arginine (31). Ornithine is the immediate precursor

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for polyamine synthesis, which is essential to proliferation, differentiation, and repair of intestinal epithelial cells (14, 20). Glutamine is known to stimulate the activity of ornithine decarboxylase (ODC), a key enzyme for polyamine synthesis, in porcine intestinal epithelial cells (15). However, the effects of ornithine and polyamines on intestinal epithelial Hsp regulation are unknown. In the present study, we demonstrate that conversion of ornithine to polyamines (putrescine, spermidine, and spermine) is essential for mediating the glutamine-dependent induction of the Hsp response in intestinal epithelial cells. In addition, ornithine and polyamines as well as glutamine facilitate stress-induced binding of HSF-1 to HSE, while having no effect on the nuclear localization of HSF-1.

MATERIALS AND METHODS

Chemicals. Chemicals were obtained from Fisher Scientific (Hanover Park, IL) unless otherwise stated. Media and all cell culture supplements were obtained from Invitrogen (Grand Island, NY), ornithine and polyamines (putrescine, spermidine, and spermine) from Sigma-Aldrich (St. Louis, MO), and α-difluoromethylornithine (DFMO) from Axxora (San Diego, CA).

Cell culture. The normal, diploid, rat small intestinal epithelial cell line IEC-18 (CRL-1589; ATCC, Manassas, VA) was used between passages 19 and 30, and grown in high-glucose (4.5 g/l) Dulbecco’s modified Eagle’s medium (DMEM) containing 2 mM l-glutamine, 5% (vol/vol) FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.1 U/ml insulin. For most experiments, 90% confluent IEC-18 cells were then incubated for 24 h in glutamine-free, reduced-serum [1% (vol/vol)] DMEM containing designated concentrations of glutamine, ornithine, or polyamines with all other supplements followed by heat shock. DFMO (5 mM) was added to 70% confluent IEC-18 cells in reduced-serum DMEM containing 0.7 mM glutamine. After 24 h, designated concentrations of polyamines were added in the medium, and incubated for 24 h followed by heat shock. Cells were heat shocked at 42°C in a water bath for 30 min. For Western blot analysis, cells were harvested after returning to 37°C for 2 h to allow Hsp70 and Hsp25 protein synthesis, whereas for real-time RT-PCR analysis, cells were scraped promptly after heat shock.

Western blot analysis. Whole cell lysates were generated as previously described (31). Cytosolic and nuclear extracts were generated using the NE-PER kit (Pierce, Rockford, IL) according to the manufacturer’s instructions. Western blotting was carried out as previously described (31). All primary antibodies were obtained from Stressgen/Assay Designs (Ann Arbor, MI). Immunoblotting of the membrane was performed using rabbit anti-Hsp25 polyclonal antibody (SPA-815), mouse anti-Hsp70 monoclonal antibody (SPA-810), rat anti-Hsc73 monoclonal antibody (SPA-815), or rabbit polyclonal anti-heatin shock factor-1 (PA1–14202; Thermo Pierce) overnight at 4°C. Membranes were rinsed with 99.5% ethanol, air-dried, and dissolved in diethylpyrocarbonate (DEPC)-treated water. Complementary DNA was synthesized using the PrimeScript RT reagent kit (TAKARA BIO, Ohtsu, Japan) from definite (500 ng) RNA. The forward and reverse primers are as follows: for rat Hsp25 (GeneBank accession no. NM_031971.1), 5′-CCTTTGGAGAGCAAACTTCTC-3′ and 5′-AGTACCTTCCACTGCTCATCCTG-3′ for rat Hsp70 (GeneBank accession no. NM_031971.1) 5′-AAGCAGACCGACGCAT-3′ and 5′-GGCGAGTGATCTCCACCTTG-3′, and for rat Hsp25 (GeneBank accession no. NM_071008.3): 5′-GGCAGCAGTCAAGCTGAGAATG-3′ and 5′-ATGTTGGTGAGGCGCTGTA-3′. The expected sizes of the PCR products for Hsp25, Hsp70, and GAPDH were 113, 107, and 143 bp, respectively. All primers were obtained from TAKARA BIO. Real-time PCR was performed in a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA) using SYBR Premix Ex Taq II (TAKARA BIO). The two-step quantification cycling protocol was used. The threshold cycle (Ct) value is defined as the cycle number at which the fluorescence crosses a fixed threshold above the baseline. As a relative quantitation, fold changes were measured using the ΔΔCt method. For each sample, the Ct value of Hsp mRNA was measured and compared with the GAPDH endogenous control as ΔCt (ΔCt = Ct.Hsp - Ct.GAPDH).

Electromobility shift assay. Electromobility shift assays (EMSA) were carried out using whole cell extracts as previously described (33). The oligonucleotide used contains four tandem inverted repeats of the heat shock element (5′-nGaAAn-3′). EMSA reactions were resolved on 4% PAGE also as previously described (33).

Data analysis. All experiments were repeated at least three times with cells of different passage numbers. Densitometry of autoradiography images was performed using ImageJ (Research Services Branch, National Institute of Mental Health, Bethesda, MD) and normalized to the signal intensity of Hsc70 for equal protein loading control for each sample in each experiment. Statistical analysis was performed by using Tukey’s honest significant difference method with SPSS 15.0J (SPSS, Chicago, IL). Data were expressed as means ± SE.

RESULTS

Ornithine and polyamines facilitate heat induction of Hsp70 and Hsp25 in glutamine-deprived IEC-18 cells. Glutamine at the physiological concentration normally found in plasma (0.7 mM) augmented heat-induced Hsp70 and Hsp25 expression at both protein and mRNA levels as previously observed (31, 33), although it had little effect in nonstressed control cells (Fig. 1). Hsc70, a constitutive member of the Hsp family, was used as a loading control and for normalizing Hsp70 and Hsp25 expression for Western blot analysis, since it is not affected by heat shock or glutamine depletion. We had previously reported that glutamine’s effect on heat-induced Hsp25 expression was the result of glutamate synthesis, but not involving conversion to glutathione. In addition, glutamate’s metabolites, such as aspartate, alanine, and praline, were not involved in glutamine’s effects on the heat shock response (31).

These data suggested alternative downstream pathways for glutamine’s actions. Glutamate can also be converted to α-ketoglutarate, ornithine, and subsequently to citrulline. Among these glutamate metabolites, physiological concentrations of ornithine (50–100 μM) enhanced the heat-induced expression of Hsp70 and Hsp25 in glutamine-deprived IEC-18 cells, although the latter to a lesser extent (Fig. 2). Ornithine had little effect on basal Hsp expression, and α-ketoglutarate and citrulline did not enhance basal or heat-induced expression of Hsp70 or Hsp25 (data not shown).

To examine the effect of ornithine on the pathway for HSF-1 activation during heat shock, we determined the cellular distribution of HSF-1. Heat stress induced HSF1 translocation from the cytoplasm to the nucleus regardless of the presence or absence of glutamine or ornithine (Fig. 3, A and B). We do not observe a shift of HSF-1 molecular mass after heat activation, similar to the manufacturer’s specifications for the HSF-1 antibody. Thus the lack of a heat shock response in the absence of glutamine cannot be explained by any changes in HSF-1 nuclear localization. We next examined the effects of glutamine and ornithine on HSF-1/HSE binding by EMSA. The
specificity of the HSF-1/HSE oligonucleotide has been demonstrated previously (33). Glutamine increased DNA binding of HSF-1 in heat-shocked cells (Fig. 3C). In heat-shocked cells in the absence of glutamine, however, ornithine was able to rescue the heat-induced DNA binding of HSF-1 (Fig. 3D). These data therefore support a role for ornithine conversion to polyamines as an essential process for glutamine-dependent Hsp induction. Interestingly, polyamines at a higher concentration, above 1 mM, failed to enhance the heat-induced Hsp expression (data not shown). This appears to be due to toxicity of exogenous polyamines in high concentrations (23). High levels of intracellular polyamines may result in cell injury, in part through amine oxidase activities. For this reason, high levels of polyamines negatively feedback to limit ODC activity (23).

Polyamines restore the defective heat shock response caused by DFMO treatment. To clarify the involvement of polyamines in glutamine’s effect on the heat shock response, IEC-18 cells were grown in the presence or absence of 5 mM DFMO, a specific inhibitor of ODC that is the first rate-controlling enzyme in polyamine biosynthesis, for 2 days. In previous studies, DFMO treatment depleted IEC-6 and Caco-2 cell putrescine and spermidine within 2 days, whereas the spermine level was reduced to 50 and 80% in the two cell lines, respectively, compared with control by day 2 (32). Polyamine depletion by DFMO reversed the effect of glutamine on the expression of Hsp70 and Hsp25 in heat-stressed cells. More-
over, exogenous addition of polyamines to DFMO-containing medium for 24 h restored the defective heat shock response (Fig. 5). The inhibition of Hsps by DFMO treatment was also seen in IEC-18 cells at a higher concentration of glutamine (2 mM), which was rescued by exogenous polyamines (data not shown). As is the case with glutamine and ornithine, DFMO and polyamines did not modify HSF-1 nuclear translocation (Fig. 6A). However, DFMO dramatically reduced HSF-1/DNA binding in the presence of glutamine. Exogenous polyamines rescued the binding of HSF-1 to HSE in DFMO-treated cells (Fig. 6B). DFMO has not been demonstrated to have few, if any, effects outside of blocking ODC and decreasing polyamines. The ability of polyamines to rescue the heat shock response after DFMO indicates that the action of DFMO under these conditions is most likely through the depletion of polyamines. Moreover, the rescue of the heat shock response by polyamines provides further support that glutamine-dependent induction of Hsp70 and Hsp25 is mediated by polyamine synthesis through the action of ODC.

**DISCUSSION**

Polyamines are ubiquitous low-molecular-weight aliphatic cations implicated in a large number of cellular processes, including ion channel function, nucleic acid packaging, DNA replication, apoptosis, transcription, and translation (2, 24).

Polyamines are essential to growth, differentiation, and migration of intestinal epithelial cells and may play an important role in regulating intestinal maturation and remodeling (14, 20). In this study, we report that polyamines mediate glutamine-dependent induction of the intestinal epithelial heat shock response via enhancement of HSF-1/DNA-binding activity.

Pathways of polyamine synthesis in intestinal epithelial cells are shown in Fig. 7. Glutamine has been shown to stimulate the expression and activity of ODC in intestinal epithelial cells (15), and glutamine is required for the conversion of proline-derived pyrroline 5-carboxylate into ornithine (45). Because other amino acids, such as glutamine and arginine, can be converted to polyamines, they can potentially contribute to the ability of intestinal epithelial cells to mount a heat shock response under conditions of stress. However, we previously demonstrated that the uptake of glutamate and arginine by intestinal epithelial cells appears to be much less than that for glutamine. Glutamate’s effect on the heat shock response in the absence of glutamine could only be enhanced by increasing their extracellular concentration and the length of incubation (31). We do not know whether increasing the concentration or length of incubation for arginine, another potential source for polyamine generation, would have a similar effect.

The extent to which polyamines mediate the physiological and protective effects of glutamine in other models of intestinal...
We have used only a physiological concentration of 0.7 mM glutamine, and glutamine is used at higher concentrations pharmacologically that may activate different pathways.

We previously reported no differences in the phosphorylation level of HSF-1 during heat shock in glutamine-depleted cells (33). While polyamines have been shown to activate casein kinase 2, which phosphorylates HSF-1 after heat shock (11, 37), our results suggest that this may not be the mechanism of action of polyamines to maintain the heat shock response in IEC-18 intestinal epithelial cells.

Fig. 5. α-Difluoromethylornithine (DFMO) inhibits glutamine-dependent heat induction of Hsp70 and Hsp25. IEC-18 cells were cultured in glutamine-deprived medium with 100 μM putrescine (Put), 50 μM spermidine (Spd), or 200 μM spermine (Spm) for 24 h followed by heat shock. The expressions of Hsp70 and Hsp25 are shown by Western blot analysis (A) and real-time PCR (B). Densitometry means ± SE of Western blot analysis are normalized to heat-shocked cells without glutamine and polyamine (arbitrarily set at 100 units). *P < 0.05 and **P < 0.01 compared with conditions without glutamine and polyamine. Hsc70 serves as a loading control. Results are representative of 4 separate experiments.

Fig. 4. Polyamines enhanced the heat-induced expression of Hsp70 and Hsp25 in glutamine-deprived cells. IEC-18 cells were incubated in glutamine-deprived medium with 100 μM putrescine (Put), 50 μM spermidine (Spd), or 200 μM spermine (Spm) for 24 h followed by heat shock. The expressions of Hsp70 and Hsp25 are shown by Western blot analysis (A) and real-time PCR (B). Densitometry means ± SE of Western blot analysis are normalized to heat-shocked cells without glutamine and polyamine (arbitrarily set at 100 units). *P < 0.05 and **P < 0.01 compared with conditions without glutamine and polyamine. Hsc70 serves as a loading control. Results are representative of 4 separate experiments.

Mucosal stress is not well understood. However, with regard to the glutamine-dependent heat shock response, the involvement of polyamines appears to be pivotal. A role for polyamines in the heat stress response has been reported in bacteria (22), plants (18), and rat hepatocarcinoma FAO cells (4, 5). In FAO cells, however, DFMO treatment reduces HSF-1/DNA binding by only 25%. In our studies, DFMO abolished the HSF-1 oligo/HSF binding completely, which may reflect differences in the activation of Hsp pathways between normal and transformed cells or between different tissue types. Although the specificity of DFMO in inhibiting polyamine synthesis might be questioned, the ability of polyamines to rescue cells after ODC inhibition by DFMO supports this site of action. Other mechanisms for glutamine maintenance of the response, including the O-Glc-Nac pathway (12), may play a role. This may be concentration dependent, since in the present studies
Polyamines have been reported to affect the function of several transcription factors by modulating their DNA binding and by interaction with DNA-binding proteins required for transcription. Spermine facilitates the binding of estrogen receptor-α and NF-κB to their DNA response elements via interaction with the CBP/p300 protein (35). Polyamines also enhance DNA-binding activity of several gene-regulatory proteins, including ICP-4, TFE3, Ig/EBP, NF-1L6, and YY1 (28).

**Fig. 7. Pathways of polyamine synthesis in intestinal epithelial cells.** Glutamine is degraded to glutamate, which is a substrate for pyrroline 5-carboxylate and subsequently for ornithine and polyamines. Glutamine also stimulates the expression and activity of ornithine decarboxylase (ODC). Polyamines enhance HSF-1/HSE binding.

Heat shock-stimulated gene transcription is also modulated by Sp-1 binding, which has been shown to be enhanced by polyamines in IEC-6 and other cell types (30). Therefore, the glutamine-dependent heat shock response may be in part mediated by polyamine enhancement of Sp-1 binding, resulting in increased Hsp gene transcription.

Polyamines might also modulate gene transcription by bending DNA (21). Molecular modeling studies have illustrated the versatility of natural and synthetic polyamines in bending DNA (8). Polyamines promote DNA bending by neutralizing the negative charges on DNA phosphate residues, reducing the energy requirement for bending, and thus facilitating enhanced protein-DNA interactions. Polyamines can also regulate DNA-binding activity of the proteins like HSF-1 by bending DNA.

In conclusion, we define a key role of polyamine synthesis in mediating the glutamine-dependent Hsp response in intestinal epithelial cells. Glutamine is needed as a substrate for the synthesis of polyamines, which in turn facilitates HSF-1 binding to HSE. Although the induction of the intestinal epithelial heat response is essential for maintaining intestinal homeostasis (13, 40), the depletion of glutamine during many conditions of stress can potentially impair this ability, resulting in enhanced severity and extent of intestinal injury.

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**DISCLOSURES**

None of the authors have any conflicts of interest to disclose.
REFERENCES