Induction of the stress response increases interleukin-6 production in the intestinal mucosa of endotoxaemic mice

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ABSTRACT

Previous studies suggest that production of interleukin-6 (IL-6) is increased in the intestinal mucosa during sepsis and endotoxaemia. We tested the hypothesis that mucosal IL-6 production during endotoxaemia is increased further by the heat-shock (stress) response. The stress response was induced in mice by hyperthermia (rectal temperature of 42 °C for 3 min) or by intraperitoneal injection of sodium arsenite (10 mg/kg). At 2 h after induction of the stress response, groups of mice were injected subcutaneously with endotoxin (10 mg/kg) or sterile saline. IL-6 mRNA and protein levels in the jejunal mucosa were determined by an RNase protection assay and an ELISA respectively, and levels of hsp72 (heat-shock protein of 72 kDa) were determined by Western blot analysis. Hyperthermia and sodium arsenite increased hsp72 levels in the intestinal mucosa. IL-6 concentrations were increased in the jejunal mucosa of endotoxaemic mice, and this effect of endotoxaemia was potentiated by the stress response. Mucosal IL-6 mRNA levels were increased in endotoxaemic mice, and were increased further by the stress response. Thus it is concluded that mucosal IL-6 production during endotoxaemia may be further stimulated by the stress response. Increased IL-6 levels in the intestinal mucosa may be a potential mechanism by which the stress response exerts a protective effect during sepsis and endotoxaemia.

INTRODUCTION

There is increasing evidence that the intestinal mucosa participates in the inflammatory and metabolic responses to sepsis and endotoxaemia [1]. In previous studies, mucosal production of acute-phase proteins was increased in septic and endotoxaemic mice [2] and in cytokine-stimulated enterocytes [3,4]. In other reports, the synthesis of cytokines, including interleukin-1 (IL-1) [5] and IL-6 [6], was increased in the gut mucosa during sepsis and endotoxaemia. Among these products, IL-6 is of particular interest, because it may act as an anti-inflammatory cytokine [7], stimulating the synthesis of acute-phase proteins, both in the liver [8] and in the enterocyte [9], and down-regulating the production of pro-inflammatory cytokines [7,10]. In addition, there is evidence that IL-6 exerts a protective effect and improves survival in various models of septic shock [11,12].
Although cells in the lamina propria contribute to mucosal IL-6 production, the enterocyte is a major source of this cytokine in the mucosa of stressed animals [13].

The heat-shock response is characterized by the rapid synthesis of a specific group of proteins called the heat-shock proteins, the most highly inducible of these being hsp72 (heat-shock protein of 72 kDa) [14]. Originally described in cells subjected to hyperthermia, the heat-shock response can also be induced by other stimuli, such as ischaemia, viral agents, ionizing radiation and certain chemicals, including sodium arsenite, and hsp72 (heat-shock protein of 72 kDa) [14]. Originally described in cells subjected to hyperthermia, the heat-shock response can also be induced by other stimuli, such as ischaemia, viral agents, ionizing radiation and certain chemicals, including sodium arsenite, and hsp72 (heat-shock protein of 72 kDa) [14]. Originally described in cells subjected to hyperthermia, the heat-shock response can also be induced by other stimuli, such as ischaemia, viral agents, ionizing radiation and certain chemicals, including sodium arsenite, and hsp72 (heat-shock protein of 72 kDa) [14]. Originally described in cells subjected to hyperthermia, the heat-shock response can also be induced by other stimuli, such as ischaemia, viral agents, ionizing radiation and certain chemicals, including sodium arsenite, and hsp72 (heat-shock protein of 72 kDa) [14].

In a recent study from our laboratory, IL-6 production in stimulated cultured enterocytes was augmented in cells expressing a heat-shock response [21]. Because of the anti-inflammatory properties of IL-6 [7], the increased synthesis of this cytokine may be one of the mechanisms by which the stress response confers a protective effect in inflammatory conditions such as sepsis. It is not known if the stress response results in increased production of IL-6 in the intestinal mucosa in vivo as well. The present experiments were designed to test the hypothesis that the stress response is associated with increased mucosal IL-6 production during endotoxaemia in mice.

**MATERIALS AND METHODS**

**Experimental design**

Male A/J mice (20–27 g) were purchased from Jackson Laboratory (Bar Harbor, ME, U.S.A.) and housed at a temperature of 22 °C in a room with a 12 h light/dark cycle for 1 week before experiments. Mice were randomly allocated to one of four groups: (1) control (no stress response) plus saline; (2) control plus endotoxin (lipopolysaccharide (LPS)) injection; (3) stress response plus saline; (4) stress response plus LPS. At different time points after induction of the stress response or injection of LPS, IL-6 protein and mRNA levels and hsp72 protein levels were determined as described below. The stress response was induced by hyperthermia or by injection of sodium arsenite.

When hyperthermia was used to induce the stress response, the protocol described by Stojadinovic et al. [22] was followed, with minor modifications. In brief, mice were anaesthetized with ketamine (60 mg/kg intramuscular) and xylazine (2.5 mg/kg intramuscular) and placed in a heated (45–47 °C) chamber. Body temperature was measured with a rectal probe (YSI-423; Yellow Springs Instrument Co, Yellow Springs, OH, U.S.A.) and monitored with a five-channel thermistor thermometer (model 8502-16; Cole Parmer Instrument Company, Chicago, IL, U.S.A.) at 1-min intervals. After the rectal temperature had reached 42 °C, the mice were kept in the heated chamber for an additional 3 min, whereafter they were removed from the chamber and placed on a heating pad (37 °C) to allow for passive cooling. The rectal temperature typically reached 40 °C within 10 min, whereafter the mice were returned to their cages. Control mice were anaesthetized and kept at room temperature.

In other groups of mice, the stress response was induced by the intraperitoneal injection of 10 mg/kg sodium arsenite (Sigma Chemical Co., St. Louis, MO, U.S.A.). Control mice were injected with a corresponding volume of solvent (sterile saline). The dose of sodium arsenite used here was based on a previous study in which maximal induction of hsp72 was found in lung tissue after injection of 10 mg/kg sodium arsenite in rats [15], and on preliminary experiments in our laboratory in which different doses of sodium arsenite (6, 8 and 10 mg/kg) were tested; maximal induction of hsp72 in the jejunal mucosa was found after injection of 10 mg/kg drug (results not shown).

At 2 h after removing the mice from the hyperthermia chamber or 2 h after injection of sodium arsenite, groups of mice were injected subcutaneously with 10 mg/kg LPS (*Escherichia coli* endotoxin 0111:B4; Calbiochem, La Jolla, CA, U.S.A.) dissolved in 0.5 ml of sterile saline, or with the same volume of sterile saline alone. Then, 4 h after injection of LPS or saline, the mice were anaesthetized (40 mg of pentobarbital, intraperitoneal) and blood was collected by heart puncture for determination of plasma IL-6 levels. A 10 cm segment of the jejunum was excised, the lumen was rinsed with ice-cold saline and the intestine was opened along the antimesenteric border. Mucosa was harvested by scraping with a microscope slide, frozen in liquid nitrogen and stored at −70 °C for subsequent measurement of IL-6 and hsp72 levels. Plasma and mucosal IL-6 levels were measured by ELISA (Endogen, Cambridge, MA, U.S.A.) according to the manufacturer’s guidelines. The lower limit of detection was 30 pg/ml. For determination of IL-6 mRNA levels, mucosa was harvested 1 h after injection of LPS or saline. IL-6 mRNA was determined as described below. The time points for measurement of IL-6 mRNA and protein levels used here were based on a previous study in which mucosal IL-6 mRNA was increased 1 h after injection of 10 mg/kg LPS in mice, and circulating and mucosal IL-6 levels were increased 4 h after injection of LPS [6]. The dose of endotoxin used here is non-lethal up to 24 h, but large enough to give rise to changes in mucosal IL-6 production ([6] and the present study). This dose was used because the purpose of the present study was to examine the influence of the...
stress response on mucosal IL-6 production during endotoxaemia, rather than survival rates.

All experiments were performed and the animals were cared for according to the National Research Council’s Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the University of Cincinnati Institutional Animal Care and Use Committee.

**Western blot analysis of hsp72**

Mucosal samples were ultrasonicated for 20 s in PBS (pH 7.4) containing 2 μg/ml each of the protease inhibitors leupeptin, aprotinin, pepstatin A and antipain (Sigma) and 2 mM PMSF (Sigma), and then centrifuged at 12000 g at 4 °C for 30 min. The protein concentration in the supernatant was determined using the Bio-Rad Protein Assay (500-0006; Bio-Rad Laboratories, Hercules, CA, U.S.A.). Western blot analysis was performed on samples of 50 μg of protein from the supernatant using a rabbit polyclonal antibody to hsp72 as primary antibody (Stressgen, Victoria, British Columbia, Canada) and a peroxidase-conjugated donkey anti-rabbit IgG (Amer sham Life Science) as secondary antibody, as described in detail previously [21]. Blots were washed in Tris-buffered saline (0.05 M, pH 7.6) containing 0.05% Tween-20 between each application, and were incubated in enhanced chemiluminescence reagent (Amer sham Life Science) followed by exposure on X-ray film and quantification by densitometry.

**IL-6 mRNA**

RNA was isolated from mucosal samples by guanidinium thiocyanate/phenol/chloroform extraction [23] using commercially available RNAzol reagent (BiotechLab Inc., Houston, TX, U.S.A.). The concentration of RNA was determined spectrophotometrically and the purity was verified by electrophoresis on a 1.0% (w/v) agarose/formaldehyde gel and subsequent visualization by ethidium bromide staining. cDNA fragments for IL-6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by reverse transcriptase–PCR using a kit (Perkin-Elmer, Branchburg, NJ, U.S.A.), according to the manufacturer’s instructions. The sequences of the PCR primers were as follows: mouse IL-6: forward, 5’-ATG AAG TTC CTG TCT GCA ACA GAC T-3’; reverse, 5’-CAC TAG GTT TGC CG AGT AGA TCT C-3’ (638 bp); mouse GAPDH: forward, 5’-ACC ACA GTC CAT GCC ATC AC-3’; reverse, 5’-TCC ACC ACC CTG TTG CTG TA-3’ (452 bp). After purification, cDNA fragments were subcloned into pGEM3. Probes were synthesized using the bacteriophage polymerase reaction and [32P]UTP (Dupont, Boston, MA, U.S.A.) according to the manufacturer’s instructions. RNAse protection assays were performed utilizing an RPA II kit (Ambion, Austin, TX, U.S.A.), which is a modification of the solution hybridization procedure described originally by Lee and Costlow [24]. Following electrophoresis of the RNase-treated samples on 5% (w/v) polyacrylamide/urea gels, the gels were quantified by phosphorimaging (Phosphor Imaging Cassettes; Molecular Dynamics Inc., Sunnyvale, CA, U.S.A.). The signals for IL-6 mRNA were normalized to GAPDH mRNA bands on the same gel.

**Morphological studies**

In a separate series of experiments, the influence of the stress response and endotoxaemia on mucosal morphology was examined. A 5 cm segment of the jejunum was harvested from non-heat-shocked and heat-shocked mice 4 h after treatment with 10 mg/kg LPS or a corresponding volume of saline administered subcutaneously, as described above. The intestinal segment was fixed in 10% formalin, embedded in paraffin and oriented so that transverse sections of the gut were obtained. Sections (5 μm thick) were stained with haematoxylin/eosin and observed with a light microscope at a magnification of ×330. Random fields were viewed, and the villus height and crypt depth were measured for 50–60 intact villus–crypt units per animal using a computer-linked video system with Image-1/AT software (Universal Imaging Corp., Westchester, PA, U.S.A.). Only crypt–villus units sectioned so that the villus was included in the assessment. The mean villus height and crypt depth were calculated for each mouse. All slides were coded, and the observers were unaware of the group of mice from which the specimen originated.

**Statistics**

Results are presented as means ± S.E.M. ANOVA followed by Student–Newman–Keuls test was used for statistical comparisons. P < 0.05 was considered statistically significant.

**RESULTS**

There was no mortality in any of the experimental groups included in the present study. Subjecting mice to hyperthermia resulted in an approx. 3-fold increase in hsp72 levels in the jejunal mucosa 2 h after the end of hyperthermia (Figure 1). The hsp72 levels remained elevated for at least 6 h after the end of hyperthermia (i.e. 4 h after injection of LPS or saline) and were not influenced by treatment of the mice with LPS (Figure 1). These results, as well as the presence of small amounts of hsp72 in the mucosa of non-heat-shocked mice, are similar to the results reported previously by Stojadinovic et al. [22] using an almost identical heat-shock protocol.
Figure 1  Expression of hsp72 in the jejunal mucosa of non-heat-shocked (37 °C) and heat-shocked (42 °C) mice, and the effect of treatment with LPS

Upper panel: Western blots of protein from the jejunal mucosa. The first two lanes contain mucosa from mice 2 h after hyperthermia (42 °C) or a corresponding control procedure (37 °C). The last four lanes contain mucosa from mice 4 h after injection of saline or LPS. Saline or LPS was injected 2 h after heat shock or the control procedure. For further details, see the text. Lower panel: quantification by densitometry of mucosal hsp72 levels. The bars are arranged in the same order as the lanes in the upper panel. Values are means ± S.E.M. (n = 6 in each group). Significance of differences: *P < 0.05 compared with corresponding non-heat-shocked group (ANOVA followed by Student–Newman–Keuls test). AU, arbitrary units.

Figure 2  IL-6 levels in the jejunal mucosa (left panel) and plasma (right panel) of saline- and LPS-injected mice
Saline or LPS was injected 2 h after hyperthermia or the control procedure, and IL-6 levels were determined 4 h later. Values are means ± S.E.M. (n = 6 in each group). Significance of differences: *P < 0.05 compared with saline; †P < 0.05 compared with corresponding non-heat-shocked group. ND, not detectable.

Mucosal and plasma IL-6 levels were increased in endotoxaemic mice (Figure 2), similar to a previous study from this laboratory [6]. The IL-6 levels were increased further in endotoxaemic mice that had been subjected to hyperthermia, and both mucosal and plasma IL-6 levels were significantly higher in heat-shocked endotoxaemic than in non-heat-shocked endotoxaemic animals (Figure 2). Heat shock alone did not influence mucosal or plasma IL-6 levels.

The influence of endotoxaemia and preceding hyperthermia on mucosal IL-6 mRNA levels is shown in Figure 3. The mucosal concentration of IL-6 mRNA was increased 1 h after injection of LPS. The IL-6 mRNA concentration was significantly higher in endotoxaemic mice that had been subjected to heat shock than in endotoxaemic mice that had not been heat-shocked. Heat shock alone induced an approx. 60% increase in mucosal IL-6 mRNA.

In order to test whether the results described above were specific for the stress response induced by hyperthermia, we next treated mice with sodium arsenite. As described in other reports [15], treatment with sodium arsenite resulted in a stress response, with an approx. 4-fold increase in hsp72 levels in the jejunal mucosa 2 h after the injection of sodium arsenite (Figure 4). The hsp72 levels remained elevated for at least 6 h after...
Figure 4 Expression of hsp72 in the jejunal mucosa of vehicle- and sodium arsenite-injected mice, and the effect of LPS or saline injection

Upper panel: Western blots of protein from the jejunal mucosa. The two left lanes contain mucosa from mice 2 h after injection of vehicle or sodium arsenite (S.A.). The four right lanes contain mucosa from mice 4 h after injection of saline or LPS in groups of mice that had been treated with vehicle or sodium arsenite 2 h before LPS or saline injection. Lower panel: quantification of hsp72 by densitometry in the mucosa of the same groups of mice. The bars are arranged in the same order as the lanes in the upper panel. Values are means ± S.E.M. (n = 6 in each group). Significance of differences: * P < 0.05 compared with vehicle.

Figure 5 IL-6 levels in the jejunal mucosa (left panel) and plasma (right panel) from saline- and LPS-injected mice that had been treated with vehicle or sodium arsenite 2 h before injection of saline or LPS

The IL-6 measurements were performed 4 h after injection of saline or LPS. Values are means ± S.E.M. (n = 6 in each group). Significance of differences: * P < 0.05 compared with saline; † P < 0.05 compared with corresponding vehicle group.

Figure 6 Jejunal mucosa in non-heat-shocked, saline-treated (top panel), non-heat-shocked, LPS-treated (middle panel), and heat-shocked, LPS-treated (bottom panel) mice

Morphological studies were performed 4 h after injection of saline or LPS (10 mg/kg). Sections were stained with haematoxylin/eosin; initial magnification × 330.

The increase in mucosal IL-6 levels in endotoxemic mice was augmented following treatment with sodium arsenite, and this effect of the stress response was even more pronounced than that noted in mice subjected to hyperthermia (Figure 5; cf. Figure 2). The increase in plasma levels of IL-6 was augmented in endotoxemic mice that had been treated with sodium arsenite (Figure 6).
5). Sodium arsenite alone did not influence mucosal or plasma concentrations of IL-6.

In order to assess the protective effect of the stress response, morphological studies were performed with non-heat-shocked and heat-shocked mice 4 h after injection of saline or LPS. Endotoxaemia resulted in mucosal damage, characterized by a significant decrease in villus height (Figures 6 and 7). In endotoxaemic mice subjected to the stress response, the villi were somewhat broader than in control mice, and the endotoxin-induced reduction in villus height was prevented. There were no significant differences in crypt depth between the experimental groups (Figure 7).

DISCUSSION

In the present study, IL-6 concentrations were increased in the jejunal mucosa and plasma of endotoxaemic mice, and this effect of endotoxaemia was enhanced in mice expressing a stress response induced by hyperthermia or sodium arsenite. Induction of the stress response alone had no effect on mucosal or circulating IL-6 levels, suggesting that endotoxin-induced mucosal IL-6 production is potentiated by the stress response in a synergistic fashion. Because IL-6 mRNA levels in endotoxaemic mice were also increased by the stress response, it is possible that the results reflect up-regulated transcription of the IL-6 gene, although other mechanisms, such as increased mRNA stability, may account for the increase in steady-state levels of IL-6 mRNA noted here. The increase in IL-6 mRNA in the jejunal mucosa noted after induction of the stress response alone is noteworthy, given the fact that mucosal IL-6 protein levels were not increased in the same group of mice. The results may reflect reduced translational efficiency of IL-6 mRNA or increased turnover of IL-6 protein after heat shock. Further experiments are needed to differentiate between these possibilities.

The present finding of up-regulated IL-6 production in mucosa expressing the stress response is in agreement with a recent study from our laboratory in which IL-6 production in stimulated cultured Caco-2 cells was increased in cells that had been subjected to hyperthermia and in which hsp72 was induced [21]. In that study as well, the stress response resulted in increased mRNA levels for IL-6 in stimulated cells, supporting the concept that the stress response potentiates IL-6 production at the transcriptional level. It should be noted that, although IL-6 was produced in cultured Caco-2 cells (a human intestinal epithelial cell line) in our previous report [21] and another recent study from our laboratory provided evidence that the enterocyte is a major source of IL-6 in the mucosa [13], the mucosa contains a number of other cell types that may produce IL-6, e.g. lamina propria cells and intraepithelial lymphocytes [25]. Thus it is possible that the results in the present study were influenced by IL-6 production in both enterocytes and non-epithelial cells.

Although the parallel changes in mucosal and plasma IL-6 levels observed here in endotoxaemic and heat-shocked mice may suggest that circulating IL-6 originates, at least in part, from the gut, it is possible that other cells and tissues, including Kupffer cells in the liver [26], are a more important source of plasma IL-6 than the intestinal mucosa. An alternative interpretation of the results could be that the mucosal IL-6 levels reflect deposition of circulating IL-6 rather than local production of the cytokine. This is less likely, however, because in a previous study mucosal IL-6 levels remained elevated in the mucosa of endotoxaemic mice even after perfusion of the intestinal vasculature, and, in the same study, immunohistochemistry showed that IL-6 was expressed in the enterocytes of the jejunal mucosa [13]. Furthermore, increased mRNA levels for IL-6 in the mucosa of endotoxaemic mice, as noted here and in a previous report [6], support the concept that IL-6 is produced locally in the intestinal mucosa during sepsis and endotoxaemia.

Mucosal levels of hsp72 were measured in the present study because it is the most highly induced heat-shock protein and has been associated with the stress response induced by hyperthermia and treatment with sodium.
arsenite [15,22]. It is likely that other heat-shock proteins were also induced by the treatments in the present study, and it should be emphasized that the association between increased hsp72 levels and potentiated IL-6 production does not prove a cause–effect relationship. Additional experiments will be needed to test further the specific role of hsp72 in the potentiation of mucosal IL-6 production during endotoxaemia.

To our knowledge, the present study is the first report of up-regulation of endotoxin-induced mucosal IL-6 production by the stress response. In other studies, a protective effect of the stress response against oxidant, thermal and ischaemic injury has been observed in enterocytes and the gut mucosa [22,27]. The mechanisms by which the stress response protects cells against stressors are not fully understood. There is evidence that heat-shock proteins can stabilize and protect nascent peptides and facilitate the processing of damaged intracellular proteins during periods of stress; hence the term ‘molecular chaperones’ for heat-shock proteins [14,28]. Another mechanism by which the stress response may protect cells and tissues, in particular during sepsis and endotoxaemia, is via decreased production of tumour necrosis factor [29,30] and IL-1 [31], as well as reduced expression and activity of inducible nitric oxide synthase [32]. From the present findings it may be speculated that increased mucosal IL-6 levels reflect an additional potential mechanism by which the stress response exerts a protective effect in the intestinal mucosa. The reduced mucosal damage noted here in endotoxaemic mice that had been subjected to hyperthermia supports the concept that high mucosal levels of IL-6 may be protective, although additional experiments are needed to establish a cause–effect relationship between high IL-6 levels and mucosal protection under the present experimental conditions.

IL-6 is a pleiotropic cytokine that has important anti-inflammatory properties, mainly as a key regulator of acute-phase protein synthesis [8,9]. Recent studies in IL-6 knockout mice have provided evidence that an additional anti-inflammatory effect of IL-6 is to down-regulate the production of several pro-inflammatory cytokines during sepsis and endotoxaemia [7]. In other studies, IL-6 improved survival in various models of septic shock [11,12]. It should be noted, however, that although IL-6 may exert a protective effect during sepsis and endotoxaemia, other reports suggest that IL-6 may also be harmful in these conditions. For example, in studies of patients with sepsis, there was a correlation between high IL-6 levels and poor outcome [33], and results from other studies suggested that increased mucosal permeability in shock and critical illness may be linked to local production of IL-6 [34]. It is possible that the different effects of IL-6 (pro- versus anti-inflammatory) reflect different concentrations of the cytokine. Further studies are needed to define the biological role and potentially protective effect of increased mucosal IL-6 production following induction of the stress response.

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