Effect of Heat Stress on Lipopolysaccharide-Induced Vascular Permeability Change in Mice

TAIYO SUGANUMA, KAORU IRIE, EMIKO FUJII, TOSHIMASA YOSHIOKA, and TAKAMURA MURAKI

Department of Pharmacology, Tokyo Women's Medical University School of Medicine, Tokyo, Japan

Received March 11, 2002; accepted August 7, 2002

ABSTRACT

The effect of heat shock protein (hsp) induction on lipopolysaccharide (LPS)-induced increase in vascular permeability was studied in mice as a model of inflammatory mediator-induced inflammatory response. Mice were exposed to an ambient temperature of 43°C for 1 h and then returned to 23°C to recover up to 24 h. Dermal contents of hsp70 and hsp90 but not heat shock cognate protein (hsc)70 increased at 6 h after heat exposure and returned to the basal level at 24 h. LPS was injected subcutaneously at 0, 2, 4, 6, or 24 h after heat exposure. Two hours after LPS injection, vascular permeability was assessed by dermal accumulation of intravenously injected dye. LPS-induced dye leakage was reduced by 42 and 49% in heat-exposed mice after recovery for 4 and 6 h, respectively. Increases in dermal tumor necrosis factor-α (TNF-α) and prostaglandin E₂ (PGE₂) contents induced by LPS were significantly reduced in the heat-stressed mice recovered for 6 h. LPS-induced increase in cyclooxygenase-2 but not TNF-α mRNA was attenuated in heat-stressed mice. Deoxyspergualin, an inhibitor of hsc70 and hsp90, and geldanamycin, a specific hsp90 inhibitor, dose dependently reversed the inhibitory effect of heat stress on LPS-induced dye leakage and dermal TNF-α content but not PGE₂ content. These results suggest that heat stress attenuated LPS-induced vascular permeability change by inducing hsp90, leading to inhibition of TNF-α production.

An increase in body temperature induces physiological and metabolic adaptations including up-regulation of heat shock proteins (hsps) (Moseley, 1994). Some hsps protect cells against various stresses such as endotoxin and reactive oxygen species (Bellmann et al., 1995; Wong et al., 1996). An anti-inflammatory function of hsps has been suggested (Anderton et al., 1993), but this role has not been studied in a model of inflammatory mediator-induced vascular permeability change.

Events in endotoxemia include an increase in vascular permeability to macromolecules (McCuskey et al., 1996). When given intradermally or subcutaneously (s.c.), lipopolysaccharide (LPS) induces plasma leakage in the skin of mice and rats (Fujii et al., 1996; Iuvone et al., 1998). This increase in vascular permeability is mediated by many proinflammatory mediators such as cytokines, eicosanoids, histamine, and nitric oxide (Fujii et al., 1996, 1997; Iuvone et al., 1999). In inflammatory cells such as peritoneal macrophages, an induction of hsp70 inhibits the synthesis of proinflammatory cytokines such as TNF-α and interleukin-1β after treatment with LPS (Ensor et al., 1994).

There is some controversy over the effect of heat stress on the action of LPS. Hotchkiss et al. (1993) reported that acute exposure to high ambient temperature protected rodents against an otherwise lethal dose of bacterial endotoxin in vivo, whereas Kluger et al. (1997) reported that heat stress enhanced LPS-induced fever. Chen et al. (2001) reported that hyperthermia increased hsps in some organs and attenuated hypotension in anaphylactic rats. In addition, King et al. (2002) reported that whole-body hyperthermia-induced thermotolerance was associated with the induction of hsp70 in mice. They showed the correlation between thermotolerance and induction of hsp70 by measuring the hepatic antioxidant activities. Although these studies suggest that heat stress modulates the process of inflammation, the mechanism(s) of the anti-inflammatory effect of heat exposure is unknown. The immunosuppressant deoxyspergualin (DSG) specifically inhibits some hsps (Nadler et al., 1992) and accelerated the suppressive effect of dexamethasone on paw edema (Oyanagui, 1996). Geldanamycin (GA) has been used as a specific hsp90 inhibitor in vivo (Bender et al., 1999) and in vitro (Czar et al., 1997) studies. In the present study, we examined whether whole-body hyperthermia decreases LPS-induced macromolecular leakage that is associated with...
TNF-α and PGE₂ production in the mouse skin. The role of hsp5 was further evaluated using DSG and GA.

Materials and Methods

Animals. Male ddY strain mice (Sankyo Laboratory Service, Tokyo, Japan) weighing approximately 35 g were used. Mice were housed in an air-conditioned room (temperature 23 ± 1°C, humidity 55 ± 5%) with a controlled light/dark cycle (light on between 6:00 AM and 8:00 PM), and food and water were available ad libitum.

Drugs. LPS (from Salmonella typhimurium, L6511) and GA were obtained from Sigma-Aldrich (St. Louis, MO), DSG was obtained from Nippon Kayaku (Tokyo, Japan), and pontamine sky blue 6B (PSB) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). LPS was dissolved in phosphate-buffered saline (PBS) adjusted to pH 7.0, GA was dissolved in 0.9% NaCl containing 1% dimethyl sulfoxide, and other drugs were dissolved in 0.9% NaCl.

Experimental Protocol. Heat stress was given by placing mice in an ambient temperature of 43°C for 1 h. To minimize circadian variations, mice were exposed to heat between 9:00 and 10:00 AM in all experiments and rectal temperature was monitored with a thermistor probe (KN-90; Natsume, Tokyo, Japan). After 1 h exposure to heat, the mice were returned to the original cages at 23°C and allowed to recover for 0, 2, 4, 6, or 24 h before LPS was injected subcutaneously. Dye leakage was determined 2 h after LPS administration as described below. Control mice were kept in an ambient temperature of 23 ± 1°C throughout the study. The experimental protocol was approved by the institutional animal care committee.

Assessment of Vascular Permeability Induced by LPS. Vascular permeability was assessed by extravasation of PSB (Murakii et al., 1996). Five minutes after an i.v. injection of PSB (50 mg/kg), LPS (0.4 mg/site) or saline (0.1 ml/site) was administered s.c. into the back of a mouse at one site per animal. Two hours after the LPS or saline injection, the mouse was killed by cervical dislocation. The stained area of the skin was excised, weighed, and minced. The minced tissue was dispersed in 6 ml of 0.5% sodium sulfate and mixed with 14 ml of acetone to extract the dye for 3 h. PSB concentration in the extract was colorimetrically determined at 590 nm. To examine the effects of hsp inhibitors, DSG (1, 5, or 10 mg/kg) or GA (0.1, 0.3, or 1.0 mg/kg) was administered i.p. 2 h before i.v. injection of PSB. The doses of DSG and GA were chosen according to previous studies (Oyanagui, 1996; Bucci et al., 2000).

PGE₂ Level in Mouse Skin. Two hours after LPS injection, the skin at the site of injection was harvested. Approximately 500 mg of tissue was immediately weighed, frozen, and pulverized in liquid nitrogen and homogenized in ice-cold buffer (0.1 M phosphate, pH 7.4, containing 1 mM EDTA, 50% ethanol, and 10 μM indomethacin) using a glass homogenizer. The homogenate was centrifuged at 15,000g for 10 min to remove undissolved material. PGE₂ in the supernatant was purified by the method of Powell and Chan (1984). Briefly, 1 ml of supernatant was applied to a Sep-Pak column (Waters, Milford, MA) previously washed successively with 5-ml portions of ethanol and distilled water. Then the column was washed with 5 ml of distilled water followed by 5 ml of hexane, and finally eluted with 5 ml of ethyl acetate containing 1% methanol. The eluate was evaporated to dryness by vacuum centrifugation, and the PGE₂ level was assayed with an enzyme-linked immunosorbent assay kit (Cayman Chemical, Ann Arbor, MI).

TNF-α Level in Mouse Skin. Approximately 500 mg of pulverized skin was weighed and homogenized in 0.5 ml of PBS containing 0.5% bovine serum albumin and 0.1% Tween 20 using a glass homogenizer. The homogenate was then centrifuged at 10,000g for 1 h at 4°C; TNF-α in the supernatant was determined using an TNF-α enzyme-linked immunosorbent assay kit (BioSource International, Camarillo, CA).

RT-PCR Analysis for the Expressions of TNF-α and COX-2 mRNA. Tissues were homogenized in ISOGEN (Wako Chemical, Tokyo, Japan) and total RNA was extracted. Single-strand cDNAs were synthesized from 1 μg of total RNA using oligo(dT) priming and superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Mouse TNF-α-specific primers used in polymerase chain reaction (PCR) analysis were sense: 5′-AGTTGGCAGCACCAGTTGGGT-TGT-3’; and antisense: 5′-GCTGAGTTGGTTCCCTTCCAG-3’. Mouse COX-2-specific primers were sense: 5′-TGTTGCTGATATG-TATGCC-3’; and antisense: 5′-TCAAGGAGAATGTGCTGCTCA-3’. As a control for cDNA synthesis, β-actin-specific primers sense: 5′-CCCGATCATGTTGGAGACC-3’ and antisense: 5′-TAGCTCTCTTCCAAGGGAGGA-3’ were used. PCR was performed with a PerkinElmer 9700 Gene Amp PCR thermocycler (Foster City, CA). The PCR protocol was denaturation at 94°C for 5 min, followed by 33 cycles (for TNF-α), 28 cycles (for COX-2), or 22 cycles (for β-actin) of amplification at 94°C for 1 min, 60°C (for TNF-α), 58°C (for COX-2), or 54°C (for β-actin) for 1 min and 72°C for 1 min, with final elongation at 72°C for 7 min. The PCR products were electrophoresed in a 7% acrylamide gel and stained with ethidium bromide. Digital images of stained gels were obtained using the Fluoro-S Multimager (Bio-Rad, Hercules, CA), and the densities of bands were analyzed by a Macintosh computer (Apple, Cupertino, CA) with image-analyzing software (Molecular Analyst; Bio-Rad).

Western Blot Analysis. Approximately 100 mg of the skin specimen, epidermal skin specimen, and dermal skin specimens (O’Brien et al., 1975) were homogenized in ice-cold PBS using a glass homogenizer. The homogenized tissue was centrifuged at 600g for 10 min. Protein concentration of the homogenate was determined by the method of Bradford (1976). Each sample (10 μg of protein) was separated on a 10% sodium dodecyl sulfate polyacrylamide gel and transferred to nitrocellulose membranes. For immunoblotting, the membranes were blocked with Block Ace (Dainippon Pharmaceutical, Osaka, Japan) for 1 h. Primary antibodies against the inducible or constitutive isoforms of hsp70 (monoclonal antibody SPA-810 and SPA-815 for inducible and constitutive hsp70, respectively; Stressgen Biotechnologies, Victoria, BC, Canada) or hsp90 (polyclonal; NeoMarkers Division, Lab Vision Corp., Fremont, CA) were applied at a 1:1000 dilution for 1 h. After washing four times in PBS containing 0.1% Tween 20, appropriate secondary antibodies (peroxidase-conjugated anti-mouse, anti-rat, or anti-rabbit IgG; Amersham Biosciences Ltd., UK, Little Chalfont, Buck-
inghamshire, UK) were applied at 1:1000 dilution for 1 h. Blots were washed four times in PBS-Tween 20 for 15 min, incubated with enhanced chemiluminescence reagents (Amersham Biosciences), and exposed to an X-ray film for 5 s. Fluor-S MultiImager was used for densitometric analyses.

Statistical Analysis. Results are expressed as mean ± S.E.M. of more than five mice. Results were analyzed for statistical significance by two-way or one-way ANOVA followed by Bonferroni/Dunn's test, or Student's t test. The results of Western blot analysis were evaluated by the Kruskal-Wallis method followed by the Mann-Whitney U test.

RESULTS

Effect of Heat Stress on LPS-Induced Vascular Permeability. During 1 h of whole-body heat exposure, the rectal temperature of mice increased from the pretreatment level of 37.4 ± 0.2°C to 42.4 ± 0.6°C at 30 min, and returned to the pretreatment level at the end of 1 h of heat exposure. We previously showed that LPS-induced dye leakage in the skin reached a plateau at 2 h after LPS injection (Fujii et al., 1996); therefore, we determined the topical dye leakage 2 h after injection of LPS in the present study. In control mice not exposed to heat stress, the amount of dye leakage induced by LPS was approximately 60 µg/g of tissue throughout the observation period of 24 h (Fig. 1) as previously reported (Fujii et al., 1996). LPS-induced dye leakage was reduced by 42 and 49% in heat-exposed mice after recovery for 4 and 6 h, respectively. No suppressive effect by heat stress was observed in mice given LPS at 0, 2, or 24 h of recovery (Fig. 1).

Effect of Heat Stress on LPS-Induced PGE2 and TNF-α Production. Our previous study showed that LPS-induced dye leakage was mediated by local production of

Fig. 2. Effect of heat exposure on LPS-induced increase in TNF-α and PGE2 contents. Mice were kept in an ambient temperature of 22–24°C (open columns) (-) or 43°C (solid columns) (+) for 1 h followed by 22–24°C for 6 h, and were challenged with LPS (0.4 mg/site s.c.) (+) or saline (0.1 ml/site) (-). Skin TNF-α and PGE2 contents were determined at 2 h after LPS injection. The columns and vertical bars represent mean ± S.E.M. of six mice. **, p < 0.01 (Bonferroni/Dunn's test).

Fig. 3. RT-PCR analysis of COX-2 gene expression in mouse skin. The experimental conditions are the same as in Fig. 2. Total RNA was extracted from skin specimens obtained 2 h after LPS injection. The COX-2 and β-actin mRNA were amplified by RT-PCR. The amounts of COX-2 mRNA were quantified by a Fluor-S MultiImager, and fluorescence intensities were normalized to those of β-actin mRNA. Typical bands are shown at the bottom. Columns and vertical bars are mean ± S.E.M. of three observations. *, p < 0.05 (Mann-Whitney U test).
several proinflammatory mediators, including PGE$_2$ and TNF-$\alpha$. To examine the mechanism of inhibition of dye leakage by heat stress, we determined the dermal contents of PGE$_2$ and TNF-$\alpha$. The LPS-induced increase in dermal TNF-$\alpha$ content was abolished by the heat stress, although heat stress moderately increased the basal TNF-$\alpha$ level (Fig. 2A). The dermal PGE$_2$ content in the LPS-treated mice increased by 2-fold compared with saline-treated mice. Heat stress inhibited the LPS-induced increase in PGE$_2$ contents without affecting the basal PGE$_2$ level.

Effect of Heat Stress on LPS-Induced Dermal TNF-$\alpha$ and COX-2 mRNA. To examine the effect of heat exposure on the transcription of TNF-$\alpha$ and COX-2 genes, dermal contents of TNF-$\alpha$ and COX-2 mRNA at the site of injection of LPS were determined. In mice without heat exposure, mRNAs of both TNF-$\alpha$ and COX-2 increased approximately five times compared with saline-treated mice at 2 h after LPS injection. In the heat-pretreated mice, however, LPS-induced increase in COX-2 mRNA was attenuated (Fig. 3). On the other hand, LPS-induced TNF-$\alpha$ mRNA increase was not altered by heat exposure (Fig. 4).

Expression of hsp70 and hsc70 in Mouse Skin after Heat Exposure. Low levels of hsp were demonstrated in the skins of control mice not subjected to heat stress. The amounts of hsp70 and hsp90 in the skin increased approximately 3-fold, but the hsc70 content was unchanged at 6 h after heat exposure (Fig. 5), at the time when LPS-induced dye leakage was attenuated. There was no increase in content of these hsps at 2 h (data not shown) or 24 h after heat stress. As shown in Fig. 6, hsp70 was induced in both dermis and epidermis, whereas hsp90 was induced in dermis after the heat treatment.

Effect of DSG and GA on Heat Stress-Induced Suppression of Vascular Permeability. The temporal association between increases in dermal hsp contents and inhibition of LPS-induced dye leakage in heat-exposed mice suggested that an induction of hsps might be related to the suppression of vascular permeability change. To prove this hypothesis, we examined the effect of hsp inhibitors, namely, DSG and GA, on heat stress-induced change in vascular permeability. When given to heat-exposed mice 2 h before LPS, DSG (0–10 mg/kg i.p.) dose dependently reversed the effect of heat exposure on LPS-induced dye leakage (Fig. 7A). DSG at a dose of 10 mg/kg completely abolished the effects of heat exposure on LPS-induced dye leakage without affecting the basal level of dye leakage (Fig. 8A). DSG had no effect on LPS-induced dye leakage in control mice without heat pretreatment (Fig. 8A). Similarly, GA (0–1 mg/kg i.p.) dose

**Fig. 4.** RT-PCR analysis of TNF-$\alpha$ gene expression in mouse skin. The experimental conditions are the same as in Fig. 2. Mice were challenged with LPS (0.4 mg/site s.c.) (+) or saline (0.1 ml/site) (-). Two hours after LPS injection, total RNA of skin specimen was isolated, and the TNF-$\alpha$ and $\beta$-actin mRNA were amplified by RT-PCR. The amounts of TNF-$\alpha$ mRNA were quantified by a Fluor-S MultiImager, and fluorescence intensities were normalized to those of $\beta$-actin mRNA. Typical bands are shown at the bottom. Columns and vertical bars are mean $\pm$ S.E.M. of three observations. *, $p < 0.05$ (Mann-Whitney U test).

**Fig. 5.** Induction of hsp70, hsc70, and hsp90 proteins in mouse skin. Mice were kept in an ambient temperature of 43°C for 1 h (+) and then allowed to recover at 22–24°C for 6 and 24 h when skin specimens were collected. Homogenates of skin (10 μg of protein/lane) were electrophoresed followed by Western blotting with specific antibodies against hsp70, hsc70, and hsp90. The densities of digital images of the blots were then determined. Typical blots are shown at the top. Columns and vertical bars are mean $\pm$ S.E.M. of three observations. *, $p < 0.05$ versus control mice (Mann-Whitney U test).
dependently attenuated the tolerance (Fig. 7B) and did not affect the basal level of dye leakage or the LPS-induced dye leakage in controls without heat pretreatment (Fig. 8B). When the effects of DSG and GA on dermal contents of TNF-α and PGE₂ were examined in these mice, DSG and GA abolished the inhibitory effect of heat stress on the LPS-induced TNF-α up-regulation. However, the effect of LPS-induced increase in PGE₂ content was not affected by either DSG or GA (Figs. 8 and 9).

**DISCUSSION**

The effects of an increase in core body temperature, or fever, remains poorly understood with respect to acute inflammatory response (Rosenberg and Gallin, 1999). Heat-exposed rodents have been shown to become tolerant to the toxic effects of LPS, as evidenced by a reduction in LD₅₀ or attenuation of increase in serum TNF-α (Hotchkiss et al., 1993, Kluger et al., 1997). Thus, whole-body hyperthermia may have an anti-inflammatory effect. A recent report in humans showed that the use of aspirin as an antipyretic prolonged illness in subjects infected with influenza A (Plaisance et al., 2000). In the present study, hyperthermia sig-
significantly attenuated LPS-induced increase in microvascular permeability in mice exposed to heat stress 4 to 6 h before LPS challenge.

The present study attempted to elucidate the mechanism of the anti-inflammatory effect of hyperthermia. Our previous study has shown that both TNF-α and eicosanoids play an important role in microvascular permeability change induced by LPS (Fuji et al., 1996; Wada et al., 2000). The present study demonstrated that heat stress suppressed LPS-induced upregulation of TNF-α and PGE₂ contents in mice to attenuate the increase in vascular permeability. Heat stress inhibited the induction of COX-2 mRNA in the skin at the LPS injection site, whereas no inhibition of TNF-α mRNA induction was observed. These results suggest that heat stress inhibits increase in PGE₂ content at the transcriptional level, whereas the stress suppresses increase in TNF-α at the post-transcriptional level. Thus, we hypothesize the presence of multiple mechanisms for the suppression of dermal vascular permeability change by heat stress.

The inhibition of LPS-induced dye leakage by heat stress correlated temporally with the induction of some hsps, namely, hsp70 and hsp90. Our results suggest that induction of hsps is related to the inhibition of LPS-induced vascular permeability change. At 6 h after heat exposure, inhibition of dye leakage was associated with increases in hsp70 and hsp90 content. At 2 h and 24 h, when no effect of heat stress on dye leakage was shown, there was no increase in dermal hsp content. hsp70 and hsp90 were induced most significantly in dermal tissue. The result was reasonable because LPS-induced vascular permeability change took place in the dermal skin. The dermal tissue is rich in fibroblasts and resident macrophages, as well as cells of blood vessels. The specific cell type that contributed to the vascular permeability change through an induction of hsps remained to be determined. In agreement with our results, Chen et al. (2001) reported that heat stress attenuated the hypotension and microvascular permeability increase in a rat model of ana-
phylaxis, and suggested the involvement of hsps in lymphocytes. LPS given i.p. has been reported to increase core body temperature to 39.5°C (Kluger et al., 1997). The LPS-induced fever may not be related to the vascular permeability change, because induction of hsps requires a core body temperature rise to higher than 41°C (Brown et al., 1985; Salminen et al., 1997), whereas LPS induces temperature increase to a lesser extent (up to 40°C).

We used DSG and GA to further examine the role of hsps in the development of tolerance to LPS by heat stress. DSG has been used as an inhibitor of hsc70 and hsp90 (Oyanagui, 1996; Bender et al., 1999), and GA specifically inhibits a function of hsp90 (Bender et al., 1996; Bender et al., 1999), whereas LPS induces temperature increase to a lesser extent (up to 40°C).

The reversal of heat-induced inhibition by DSG and GA abolished the effect of heat stress, they showed no effect on LPS-induced dye leakage in mice not exposed to heat stress. The reversal of heat-induced inhibition by DSG and GA is not due to their proinflammatory effect because DSG and GA did not increase dye leakage in non-heat-exposed mice. Although heat stress increased both the hsp90 and hsp70 contents, the contribution of hsp70 and hsp90 to the anti-inflammatory effect was unknown because there is no specific inhibitor of hsp70. The reversal of heat-mediated inhibition of LPS-induced dye leakage by DSG and GA correlated with an increase in dermal TNF-α but not PGE₂ content. Therefore, the reversal by DSG and GA may be mediated by TNF-α. Since heat stress did not alter TNF-α mRNA expression, DSG may alter the translation or degradation of TNF-α by interfering with the chaperone functions of hsps.

LPS binds to toll-like receptor 4 to increase the proinflammatory mediators through activation of NF-κB (Hwang et al., 1997; Muzio et al., 1998; Swantek et al., 1999) followed by increased transcription of the TNF-α and COX-2 genes. In preliminary experiments, when LPS (S. typhimirum) was given to C3H/HeJ, a mouse strain with mutated toll-like receptor 4, and C3H/HeN, with normal toll-like receptor 4, LPS-induced dye leakage was markedly less in C3H/HeJ than in C3H/HeN mice. Therefore, LPS used in the present study may have stimulated toll-like receptor 4. Our unpublished data showed that LPS-induced dye leakage was attenuated by pyrrolidine dithiocarbamate, an inhibitor of NF-κB activation (Muzio et al., 1998; Allport et al., 2000; Ross et al., 2000). Both hsp90 and hsp70 have been shown to inhibit NF-κB activation but by different mechanisms (Nissen and Yamamoto, 2000; Pritts et al., 2000). The effect of heat-induced hsps on the signaling of NF-κB remains to be studied.

We showed that whole-body hyperthermia in mice inhibited LPS-induced increase in vascular permeability and that DSG reversed the effect of hyperthermia. The attenuation of LPS-induced dye leakage by heat stress suggests that fever plays an important role in the protection against the pathophysiological effects of LPS. Our results suggest that heat shock protein responses are one determinant of vascular permeability change during inflammation. The effect of hyperthermia may be mediated by induction of hsps leading to inhibition of TNF-α and PGE₂ production in the skin.

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**Address correspondence to:** Taiyo Suganuma, Ph.D., Department of Pharmacology, Tokyo Women’s Medical University School of Medicine, 8-1 Kawada-cho, Shinjuku-ku, Tokyo, 162-8666, Japan. E-mail: taiyo@research.twmu.ac.jp