Lipoplysaccharide pretreatment attenuates myocardial infarct size: A possible mechanism involving heat shock protein 70–inhibitory κBα complex and attenuation of nuclear factor κB

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Objective: Lipopolysaccharide pretreatment is known to reduce myocardial infarct size, but the mechanism has not been elucidated. We hypothesized that heat shock protein 70, induced by lipopolysaccharide pretreatment, formed complexes with inhibitory κBα, thereby inhibiting degradation and attenuating activation of nuclear factor κB and cellular injury in rat myocardium.

Methods: Fifteen Sprague-Dawley rats were given saline solution (control group) or lipopolysaccharide. After 48 hours, 5 hearts in each group were excised without ischemia for examination of heat shock protein 70 and inhibitory κBα levels and detection of heat shock protein 70-inhibitory κBα complexes. Myocardium from the remaining 10 rats in each group was exposed to 30 minutes of ischemia and 30 minutes of reperfusion (n = 5) to evaluate nuclear factor κB activity or to 24 hours of reperfusion (n = 5) to evaluate infarct size.

Results: Infarct size was reduced in the lipopolysaccharide group (P < .05). Nuclear factor κB was activated in the control ischemia group and attenuated in the lipopolysaccharide group (P < .05). Heat shock protein 70 levels were increased in the lipopolysaccharide group (P < .05), but inhibitory κBα levels were similar in both groups. Heat shock protein 70–inhibitory κBα complexes were detected only in the lipopolysaccharide group. Colocalization of the 2 proteins was observed in the lipopolysaccharide group.

Conclusions: Heat shock protein 70, induced by lipopolysaccharide pretreatment, forms complexes with inhibitory κBα and attenuates activation of nuclear factor κB and myocardial infarct size. Our results suggest that attenuation of nuclear factor κB through a mechanism forming heat shock protein 70–inhibitory κBα complexes might protect the myocardium from ischemia-reperfusion injury.
pretreatment showed protection in the early phase, whereas ischemic preconditioning, heat stimuli, monophosphoryl lipid A, and interleukin 1 pretreatment have been reported to reduce ischemia-reperfusion injury and to prevent hemodynamic depression after ischemia in the delayed phase.

Lipopolysaccharide (LPS) pretreatment is also known as delayed preconditioning and is used to enhance cellular tolerance to ischemia in various organs, including the liver, kidney, brain, and heart. In LPS-treated hearts it was shown that LPS increased levels of heat shock protein 70 (HSP70), and investigators have shown that myocardial protection reduces infarct size and maintains cardiac function, which is associated with the HSP70. HSP70 functions as a chaperone, folding and transporting newly synthesized proteins and degrading damaged proteins. When expression of HSP70 is induced in cells exposed to stress, HSP70 often rescues cells from death through its chaperone function. In the myocardium HSP70 is induced 24 to 72 hours after LPS pretreatment and ischemic preconditioning. However, there is no evidence that HSP70 directly attenuates ischemia-reperfusion injury in the myocardium.

In the process of myocardial infarction, the transcription factor nuclear factor κB (NF-κB) plays a role in the regulation of cellular function. NF-κB is involved in inflammatory processes and promotes transcription of multiple depressive cytokines. NF-κB is a ubiquitous, inducible transcription factor that exists as a latent cytoplasmic form complexed primarily with p50 and p65 bound to inhibitory κB (IκB) proteins. IκB proteins consist of several subtypes, but IκBα is thought to play an essential role. Once IκBα is phosphorylated by any stimuli, it is dissociated from the most common form of the NF-κB, and IκB permits translocation into the cardiomyocyte nucleus with transcriptional regulation of multiple depressive cytokines, such as tumor necrosis factor. NF-κB translocates from the cytosol to the nucleus in a process called NF-κB activation. We hypothesized that increased levels of HSP70, which forms complexes with IκBα and inhibits its degradation, lead to attenuation of NF-κB activation and reduction in myocardial infarct size. We tested our hypothesis in hearts of LPS-treated rats.

Materials and Methods
This investigation was performed in accordance with the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996. Our protocol was also approved by the Committee of Animal Experiments at Tokyo Medical and Dental University (Principal Guideline for the Use of Laboratory Animals in Tokyo Medical and Dental University, published 1988).

Experimental Procedure
Male Sprague-Dawley rats (body weight, 250-300 g) were acclimated in a quarantine room and maintained on a standard pellet diet for 10 days without any stresses. LPS (Escherichia coli, O-127) was purchased from Sigma Chemical Company (St Louis, Mo). The experimental procedure is shown in Figure 1. We established 3 experimental groups: the sham operation group (intraperi-
tional saline solution, 2 mL; n = 5); the control group (intraperitoneal saline solution, 2 mL; n = 15); and the LPS group (intraperitoneal LPS, 3 mg/kg; n = 15). At 48 hours after LPS administration, 5 rats each in the control and LPS groups were anesthetized with ketamine (40 mg/kg administered intraperitoneally), and hearts were rapidly excised without ischemia and arrested in cold phosphate-buffered saline solution (PBS). The apex of the left ventricle was cut and prepared in OCT compound (Tissue-Tek; Sakura, Tokyo, Japan) for immunohistochemistry. The remaining left ventricle was frozen in liquid nitrogen for evaluation of the HSP70 and IκBα protein levels and for detection of HSP70-IκBα complexes. The remaining 10 rats in the control and LPS groups and 5 rats in the sham group were used in ischemia-reperfusion experiments. Each of the rats was anesthetized with ketamine, intubated, and ventilated. After left thoracotomy, the left anterior descending coronary artery was occluded for 30 minutes and reperfused either for 30 minutes for evaluation of NF-κB activation (all groups, n = 5 in each) or for 24 hours for assessment of infarct size (control and LPS groups, n = 5 in each).

To confirm whether HSP70-IκBα complexes are present in myocytes, we used neonatal rat cardiomyocytes. The cardiomyocytes were cultured and stimulated with LPS for 48 hours to detect HSP70-IκBα complexes.

**Evaluation of Infarct Size**

Infarct size was evaluated as described previously. After 24 hours of reperfusion after the 30-minute period of ischemia, hearts were excised and arrested in ice-cold PBS. The left anterior descending coronary artery was ligated, and 1% Evans blue dye solution was injected through the ascending aorta to determine the risk and nonrisk areas. The heart was cut into 2-mm-thick slices, and the second slice from the ligated zone was stained by means of incubation at 37°C for 20 minutes in 1% triphenyl-tetrazolium-chloride in PBS (pH 7.4). Slices were then scanned with a computerized scanner, and total area, nonrisk area, and infarct area were measured with Scion Image software (Microsoft Corporation, Redmond, Wash).

**Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays**

After 30 minutes of reperfusion and 30 minutes of ischemia, hearts were excised. Nuclear proteins were prepared with a modification of the method described by Dignam and colleagues and Manning and coworkers. In brief, the left ventricle of the risk area was homogenized in 5 mL of buffer A (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 10 mmol/L; MgCl2, 1.5 mmol/L; KCl, 10 mmol/L; dithiothreitol, 1 mmol/L; and phenylmethylsulfonyl fluoride, 1 mmol/L) with a Dounce homogenizer (Bellco Glass, Inc, Vineland, NJ). The samples were centrifuged at 900g for 10 minutes. The pellets were dissolved in 1 mL of buffer A and 0.1% Nonidet P-40 and centrifuged at 200g for 10 minutes to eliminate unbroken cells. The supernatant was collected and centrifuged at 900g for 10 minutes. The pellets were washed once with buffer A and purified by using the modified sucrose-gradient method.20 The pellets were dissolved in 50 μL of buffer B (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 20 mmol/L; glycerol, 25% vol/vol; NaCl, 420 mmol/L; MgCl2, 1.5 mmol/L; ethylenediamine tetra-acetic acid, 0.2 mmol/L; dithiothreitol, 1 mmol/L; and phenylmethylsulfonyl fluoride, 1 mmol/L), incubated for 45 minutes on ice, and centrifuged at 20,000g for 20 minutes. The supernatant was collected, and the protein concentrations were measured with the bicinchoninic acid method (Amersham Corp, Arlington Heights, Ill).

An electrophoretic mobility shift assay was performed to evaluate NF-κB activity, as described previously. Twenty micrograms of each nuclear protein sample was incubated with an end-labeled double-stranded oligonucleotide probe, which had the NF-κB consensus sequence 5’-AGT TGA GGG GAT CCC AGG C-3’ (Promega Corp, Madison, Wis); [γ-32P] adenosine triphosphate (3000 Ci/mmol, Amersham); and T4 polynucleotide kinase (Amersham) according to the manufacturers’ protocols. The DNA protein complexes were separated on 4% non-denaturing polyacrylamide gels, and the gels were vacuum dried and exposed to Image-Plate (Fuji Film, Tokyo, Japan) overnight at room temperature. Specific band intensities were quantified with the FLA 3000 fluoroimage analyzer (Fuji Film) and analyzed with the ImageGauge analyzing system (Version 3.12, Fuji Film).

**Western Blotting and Immunoprecipitation**

HSP70 and IκBα protein levels were examined by means of Western blotting. In brief, a small part of the excised frozen left ventricle was thawed and homogenized in 1.5 mL of buffer A with a Dounce homogenizer. After centrifugation (10,000g for 30 minutes), the supernatants were collected, and the protein concentrations were measured by using the bicinchoninic acid method. Forty micrograms of each sample was separated by means of electrophoresis on a 10% denaturing polyacrylamide gel, and the gels were vacuum dried and exposed to Image-Plate (Fuji Film). After electrophoresis, the proteins were transferred electrophoretically to polyvinylidene difluoride membrane (Bio-Rad Laboratories Inc, Hercules, Calif) overnight at 4°C. The membrane was incubated in 2% nonfat dry milk and 0.2% Tween-20 in PBS followed by incubation with specific anti-IκBα antibody (anti-rabbit polyclonal, C21, Santa Cruz, Calif) or anti-HSP70 antibody (anti-mouse monoclonal, W27; Santa Cruz). After extensive rinsing with Tween-PBS, blots were incubated with horseradish peroxidase–conjugated anti-rabbit and anti-mouse secondary antibody and developed with the enhanced chemiluminescence system (ECL kit, Amersham). The developed films were scanned with a computer scanner, and the intensities of the bands were measured with Scion Image software (Microsoft).

HSP70-IκBα complexes were detected by means of immunoprecipitation and Western blotting. In brief, 200 μg of cytosolic extracts was incubated with 20 μL of Protein G-Sepharose (Amersham). After centrifugation (10,000g for 5 seconds), 5 μg of anti-HSP70 antibody was added to the total supernatants. After a 1-hour incubation at 4°C, 20 μL of Protein G-Sepharose was added to each sample. After centrifugation (2000g for 5 minutes), the pellets were collected and washed 5 times with Buffer A. The precipitates were examined by means of Western blotting with anti-IκBα antibodies. The blots were developed with the ECL system (Amersham).

**Immunohistochemistry**

Immunofluorescent staining of IκBα with rhodamine-conjugated anti-rabbit IgG antibody (23828, Polysciences, Inc, Warrington, Pa) was performed as described previously. After staining,
immunofluorescent staining of HSP70 with FITC-conjugated anti-mouse IgG antibody (23799, Polysciences) was performed. Immunofluorescent images were obtained with a ZEISS LSM510 laser-scanning confocal microscope (Carl Zeiss Corp, Stuttgart, Germany).

**Statistical Analysis**
Results are expressed as means ± SEM. Data were analyzed by means of analysis of variance. If the analysis of variance showed an overall difference, post hoc comparisons were performed with the Bonferroni-Dunn test for paired or unpaired data, as appropriate.

**Results**

**Reduction of Infarct Size**
Representative slices of left ventricle from control and LPS rats at 24 hours of reperfusion and 30 minutes of ischemia are shown in Figure 2. The infarct area was reduced in the LPS group, and the results of these measurements are shown in Figure 3. The risk/total area ratio was similar in both groups (53.51 ± 2.90 for the control group and 54.26 ± 2.98 for the LPS group), but the infarct/at-risk area ratio was significantly less in the LSP group (31.30 ± 2.01) than in the control group (57.05 ± 0.85, P < .0001).

**Change of NF-κB Activation**
The results of using electrophoretic mobility shift assays to evaluate NF-κB activation after 30 minutes of reperfusion are shown in Figure 4. NF-κB bands in the control group were significantly more intense than those in the sham-operated group (2.35 ± 0.17 for the control group and 1.00 ± 0.10 for the sham-operated group, P = .0001). NF-κB bands in the LPS group were significantly less intense than those in the control group (1.86 ± 0.21 for the LPS group, P = .0039).

**Changes of HSP70 and IκBα Protein Levels**
At 48 hours after LPS administration and before ischemia, HSP70 and IκBα protein levels in the cytosol were examined by means of Western blotting (Figure 5). HSP70 levels in the LPS group were higher than those in the control group (1.00 ± 0.07 for the control group and 1.25 ± 0.08 for the LPS group, P = .0469). However, IκBα levels were similar in both groups (1.00 ± 0.10 for the control group and 0.98 ± 0.17 for the LPS group).

**Detection of HSP70-IκBα Immunocomplexes**
At 48 hours after LPS administration (before ischemia), immunoprecipitation and Western blotting were performed. The results are shown in Figure 6. HSP70-IκBα complexes are visible as IκBα bands. HSP70-IκBα complexes were present at detectable levels only in the LPS group (Figure 6, bottom).

Immunohistochemistry analysis showed that IκBα densities were similar in both the control and LPS groups (Figure 7, A and C), but HSP70 was stained more strongly in samples from the LPS group than in samples from the control group (Figure 7, B and D). A confocal image of a sample from the LPS group is shown (Figure 7, E). Localization of IκBα (red; Figure 7, C) and HSP70 (green; Figure 7, D) was identical, suggesting the presence of complexes,
Discussion

In the present study we investigated the role of HSP70 in attenuation of NF-κB activation in rat hearts and cultured myocytes. HSP70 expression was induced by means of LPS pretreatment, and HSP70-1κBα complexes were formed. These complexes subsequently attenuated NF-κB activation. These changes appeared to be associated with a reduction of infarct size in rat hearts after ischemia and reperfusion.

Regarding the method of measuring the risk area of the heart, we used Evans blue dye (molecular weight 960.8 d), according to the method of Yamashita and colleagues. It is criticized that dyes with a small molecule, like Evans blue, used for measuring the risk area might perfuse into the nonperfused area. However, zinc cadmium sulfide microspheres (1- to 10-μm diameter) used for this purpose resulted in a similar risk area to ours.

Resistance of cells to various forms of injury is induced by LPS, and this resistance has been studied in the liver, kidney, brain, and myocardium. In myocardium LPS pretreatment was reported to reduce infarct size in relation to HSP70 levels and to improve cardiac function after ischemia and reperfusion. Various mechanisms for the

Figure 3. Measurement of infarct area ratios (n = 5 in each group): A, average ratio of risk/total area; B, average ratio of infarct/risk area. Results are expressed as the mean ± SEM. *P < .0001 compared with control.

Figure 4. NF-κB binding activities of nuclear proteins in each group after 30 minutes of ischemia and 30 minutes of reperfusion: A, representative NF-κB band in each group; B, ratio of average intensities of all NF-κB bands in each group. The average of the sham group was considered to be 1.00. NC, Negative control (protein absent); Sham, sham operation group (no ischemia). Results are expressed as the mean ± SEM (n = 5 each group). *P = .0001 compared with sham operation group, and #P = .0039 compared with control group.
protective effects of LPS pretreatment have been proposed: (1) alteration of inflammatory mediators from monocytes and macrophages; (2) downregulation of the expression of endothelial cell adhesion receptors, which could account for decreased tissue sequestration of neutrophils; (3) effects of the NO system; and (4) increased myocardial capillary density. However, the precise mechanism underlying HSP70-associated reductions in infarct size or improvement of cardiac function is not yet well understood.

It is generally agreed that expression of HSP70 increases 24 to 72 hours after cells are exposed to various stimuli, including LPS, heat shock, and ischemic preconditioning. Our previous data showed HSP70 was detected most strongly at 48 hours compared with at 24 or 72 hours by LPS administration (data were not shown). We decided to use the condition of 48 hours. LPS was administrated intraperitoneally at 3 mg/kg, which is among the amounts used by other investigators. The levels of HSP70 after

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**Figure 5.** Levels of HSP70 and IκBα proteins in control and LPS group samples at 48 hours after LPS administration: A, representative bands and statistical analysis of HSP70; B, representative bands and statistical analysis of IκBα. Open arrowheads indicate HSP70 and IκBα bands, and filled arrowheads indicate nonspecific bands. M, The 65-kd and 45-kd markers. *P = .0469 compared with the control group.

**Figure 6.** Immunoprecipitation followed by Western blotting 48 hours after administration of LPS. The 10 different lanes correspond to each sample from 5 rats of the control group and 5 rats of the LPS group. M, The 65-kd marker. Samples were immunoprecipitated with anti-HSP70 and then examined with anti-HSP70 (A, top panel) and anti-IκBα (B, bottom panel) by means of Western blotting. In B the immunocomplex was visualized as IκBα bands only in the LPS group.
LPS pretreatment in our study were consistent with those of other studies.

HSP70 has a chaperone function, folding newly synthesized proteins, transporting them, and degrading damaged proteins. Of these functions, degradation of damaged proteins is particularly important. HSP70 is known to affect the ubiquitin-dependent proteolysis system. Most proteins in the cytosol exist in a soluble form, with hydrophilic amino
acids on the outside and hydrophobic amino acids in the core. When these proteins are damaged by various stresses, the molecular structure is changed to expose the hydrophobic residues to the exterior of the protein. The damaged proteins begin to aggregate to form insoluble pellets, and the proteins are subsequently degraded by proteolysis. During this process, HSP70 binds to the hydrophobic surface of the damaged proteins to prevent aggregation. In the reperfused ischemic myocardium, many damaged proteins might be affected by the chaperoning function.

The NF-κB activation consists of 2 processes, translocation to the nucleus and binding to DNA, and both of the 2 activation processes are controlled by redox regulation. Dissociated IκBα is polyubiquitinated by ubiquitin ligase and degraded through 26S proteasome proteolysis. Thus, NF-κB activation can be inhibited through 2 possible pathways: the reduction of various reactive oxygen species and the prevention of the IκBα degradation process. Theoretically, HSP70 might affect both pathways; however, to our knowledge, there have been no reports of attenuation of NF-κB activation by HSP70. Therefore, we focused our analysis on the pathway in which HSP70 directly affects IκBα.

In the present study IκBα protein levels were not significantly different in hearts from control and LPS group animals at 48 hours after administration of LPS. Whether IκBα levels are increased at 48 hours after LPS administration remains controversial, but our results indicate that the myocardium had attenuated NF-κB activation and increased tolerance to ischemia without increased IκBα levels. These results suggest mechanisms other than an increase in IκBα expression might exist. Thus, we hypothesized that HSP70 forms a complex with IκBα, attenuating NF-κB activity, and our experiment revealed formation of HSP70-IκBα complexes.

How the HSP70-IκBα complex acts during the process of NF-κB activation is still unclear. HSP70 might bind IκBα to prevent its phosphorylation by IκB kinase, or HSP70 might inhibit IκB kinase directly. It is also possible that the HSP70-IκBα complex inhibits the IκB kinase indirectly. Although we observed formation of complexes between IκBα and HSP70, it is still unclear whether HSP70 forms a complex with IκBα/NF-κB dimers or with IκBα protein during degradation. It is also unclear whether HSP70 affects IκBα through unknown proteins derived from pathways triggered by extracellular stimuli. Immuno-precipitation with anti-HSP70 antibody and anti–NF-κB antibody, Western blotting with anti–phosphorylated IκBα antibody, and Western blotting with anti-ubiquitin antibody should be investigated to confirm that in vivo inhibition of NF-κB translocation during myocardial ischemia-reperfusion injury is secondary to HSP70 interference with IκB.

This is the first report of IκBα stabilization because formation of complexes with HSP70 attenuates NF-κB activation. We proposed that there is cytoprotective effect of LPS by delayed preconditioning and a certain mechanism of the protection. As for LPS cytotoxicity, the signaling of LPS into the cardiomyocyte and into the myocardial resident macrophage has been well worked out. LPS is typically bound by a circulating LPS binding protein, which typically docks on toll-like receptor 4. The intracellular extension of toll-like receptor 4 binds to an adaptor protein (MYD88), which then links to interleukin 1 receptor–associated kinase, which can activate tumor necrosis factor receptor associated factor. Through a subsequent series of kinase activations, IκB is phosphorylated, and NF-κB is activated. Compared with studies of LPS cytotoxicity, the mechanism of LPS cytoprotection is not elucidated enough. Direct administration of LPS is not allowed clinically, but pharmacologic preconditioning, such as that with monophosphoryl lipid A, is known to induce delayed preconditioning effects, such as LPS. Our results suggest that attenuation of NF-κB by the mechanism of HSP70-IκBα complexes plays a clinically relevant role in the reduction of infarct size.

In conclusion, we show that HSP70 induced by LPS pretreatment forms complexes with IκBα and inhibits IκBα degradation and NF-κB translocation into the nucleus, resulting in a reduction in myocardial infarct size.

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