Endotoxin-Induced Enhancement of Angiotensinogen Synthesis in the Liver: Decreased Response Following Repeated Endotoxin Exposure

Masaoki TAKANO, Kazuyuki YOKOYAMA, Katsutoshi YAYAMA, Norio ITOH, and Hiroshi OKAMOTO*

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kobe-Gakuin University, Ikawadani-cho, Nishi-ku, Kobe 651-21, Japan. Received February 15, 1993

To understand the mechanisms responsible for lipopolysaccharide (LPS)-induced enhancement of angiotensinogen synthesis in the liver, studies were carried out in rats with repeated doses of LPS. The administration of sublethal dose (50 μ g, i.p.) of LPS to rats resulted in increase in serum levels of tumor necrosis factor (TNF) and interleukin-6 (IL-6), which attained their maximal levels by 1 and 2–4 h, respectively. Serum levels of angiotensinogen and α_2 -macroglobulin, a typical acute-phase protein in the rat, were also increased by a primary LPS challenge, and their maximal levels for the formation of TNF and IL-6 were delayed with peaks at 12 and 48 h, respectively. Repeated i.p. administration of LPS (50 μ g/d) for 5 consecutive days induced a hyporesponsiveness to its subsequent administration in terms of increasing serum TNF, IL-6 and α_2 -macroglobulin. In these LPS-tolerant rats, either LPS-induced elevation of angiotensinogen concentration in serum or angiotensinogen mRNA levels in liver was completely eliminated. Angiotensinogen synthesis in rat hepatoma H4 cells was enhanced *in vitro* by the addition of sera which had been collected 2 or 4 h after a primary injection of LPS, while not by sera collected from LPS-pretreated rats after a secondary LPS exposure. These results indicate that LPS-induced enhancement of angiotensinogen synthesis in the liver is desensitized in rats after repetitive LPS exposure, presumably by the failure of LPS-induced IL-6 production.

Keywords angiotensinogen; interleukin-6; tumor necrosis factor; lipopolysaccharide; α_2 -macroglobulin

Severe infection, particularly with gram-negative bacteria results in profound physiologic and metabolic alterations including fever, hypotension, widespread organ dysfunction, and ultimately death. The toxicity caused by gram-negative bacteria has been ascribed to lipopolysaccharide (LPS), a component of the bacterial cell wall.¹⁾

Administration of LPS to rats results in changes in the renin-angiotensin system, including decrease in plasma renin concentration and increase in plasma concentration of angiotensinogen.^{2–4)} Elevated levels of plasma angiotensinogen have also been observed in humans with acute infectious diseases.⁵⁾ Angiotensinogen is a plasma protein, predominantly synthesized in the liver, that serves as the precursor of the decapeptide angiotensin I formed by the limited proteolytic action of renin. Since angiotensinogen mRNA levels in the liver are increased in the rat by injection of LPS,⁶⁾ LPS-induced increase in plasma angiotensinogen has been ascribed to increased synthesis of this protein by the liver. In previous studies, we found that LPS-induced increase in plasma angiotensinogen is mediated by a leukocyte-derived factor,⁷⁾ and subsequently interleukin-6 (IL-6) was found to stimulate angiotensinogen synthesis *in vitro* in cultured hepatoma cells,⁸⁾ suggesting that this cytokine is the mediator responsible for LPS-induced increase in angiotensinogen synthesis.

It has long been known that repeated administration of LPS induces a state of resistance to its own effects, a phenomenon that is known as LPS tolerance. Tolerance was observed with many of the *in vivo* effects of LPS, which have been attributed to the mediation by cytokines, including tumor necrosis factor α (TNF), IL-1, and IL-6. Many investigators later demonstrated that LPS tolerance is associated with reduced productions of these cytokines.^{9–11)}

In the present study, LPS-tolerant rats were examined to evaluate the role of IL-6 in LPS-induced enhancement of

angiotensinogen synthesis. The studies demonstrated that a repeated LPS stimuli resulted in an absence of LPS-induced synthesis of angiotensinogen in the liver.

Materials and Methods

Treatment of Animals Male Sprague-Dawley SPF rats (Japan SLC, Hamamatsu, Japan), weighing 120–140 g, were used. LPS tolerance was induced by the daily i.p. injection of 50 μ g per animal of LPS (*Staphylococcus typhosa* 0901, Difco, Detroit, Mich. U.S.A.) dissolved in LPS-free saline for 5 consecutive days. The control group received the same volume of LPS-free saline. On various days after the first injection of LPS or vehicle, a blood sample (40 μ l) was collected from the tail-tip of each animal after making a slight cut with a razor. Plasma was prepared and stored at -20°C until assay. Fifteen days after the first LPS or vehicle injection, all animals received i.p. injection of LPS at a dose of 50 μ g/animal. At various times after the injection, blood was collected from the abdominal aorta into a plastic syringe under ether anesthesia. Immediately after collection of blood, liver samples (about 0.5 g) were isolated for the extraction of RNA. Serum was prepared and stored at -20°C until assay.

Assays of Angiotensinogen and α_2 -Macroglobulin in Serum Angiotensinogen was measured by radioimmunoassay (RIA) using ^{125}I -labeled rat angiotensinogen and rabbit anti-rat angiotensinogen, as described previously.⁸⁾ α_2 -Macroglobulin was also measured by RIA using ^{125}I -labeled rat α_2 -macroglobulin and rabbit antibody.¹²⁾ In these assays, duplicate estimations were made for each point of the standard curve and for unknown samples.

Assay of TNF α and IL-6 in Serum Cytolytic TNF activity in rat serum was determined by means of a TNF-sensitive murine L929 fibroblast.¹³⁾ Briefly, L929 cells were plated in a 96-well plate at a density of 1×10^4 cells/well in 100 μ l RPMI 1640 medium with 5% fetal bovine serum (FBS) and 5 μ g/ml of cycloheximide. Rat sera were assayed at final dilutions between 1:20–1:200 in these wells. After overnight incubation in a 5% CO_2 incubator, the vital cells were measured by the MTT method.¹⁴⁾ Human recombinant TNF α (3×10^6 U/mg; generously kindly supplied by Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) was used as the standard. TNF concentrations in sera were expressed as U/ml.

Serum IL-6 was measured by bioassay using an IL-6-dependent murine hybridoma clone, MH60·BSF2 cells (kindly supplied by Dr. T. Hirano, Biomedical Research Center, Osaka University Medical School, Osaka, Japan).¹⁵⁾ Cells (1×10^4 /well) were cultured in a 96-well plate with 200 μ l of RPMI 1640 medium containing 10% FBS and diluted serum samples for 72 h. Serum samples were diluted between 1:100–1:486000; then the

vital cells were measured by the MTT method. Human recombinant IL-6 (5.2×10^6 U/mg; supplied by Dr. T. Hirano) was used as the standard, and the results were expressed as U/ml.

Northern Blot Analysis of Angiotensinogen mRNA in the Liver Plasmid containing rat cDNA encoding angiotensinogen¹⁶⁾ was the gift of Dr. S. Nakanishi, Kyoto University School of Medicine, Kyoto, Japan, and the 1097-base pair *AccI* fragment of pRag16 was prepared as the hybridization probe. The cDNA fragment was radiolabeled with [α -³²P]dCTP by random priming.¹⁷⁾ Total RNA of rat liver was isolated by the acid guanidinium thiocyanate-phenol-chloroform method,¹⁸⁾ dissolved in sterile water and then the concentration was determined by absorbance readings at 260 nm. Aliquots (10 g) of total RNA were electrophoresed in a 1.2% agarose gel containing 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 1 mM EDTA, 8 mM sodium acetate (pH 4.0), and 2.2 M formaldehyde and transferred to nylon membrane. Filters were subjected to UV irradiation (4.5 J/cm²) and prehybridized at 65°C for 30 min in a hybridization buffer containing 1% bovine serum albumin, 1 mM EDTA, 7% sodium dodecyl sulfate (SDS) and 5% formamide in 0.5 M sodium phosphate, pH 7.2.¹⁹⁾ The filters were then hybridized in hybridization buffer at 65°C with ³²P-labeled cDNA for 16–18 h. Filters were rinsed at 65°C for 2 × 15 min in 40 mM sodium phosphate, pH 7.2, containing 1 mM EDTA and 1% SDS. Autoradiography was performed with X-ray films at -90°C. To demonstrate equivalent loading of RNA, filters were stripped of radiolabeled probe by boiling and rehybridized with the ³²P-labeled oligonucleotide, which recognized rat 18S rRNA.

Radiolabeling of Hepatoma Cell Cultures Reuber rat hepatoma H4IIEC3 (H4) cells (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) were grown to confluency on a 24-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS. Cells were washed with FBS-free DMEM, and then the medium was replaced with 0.5 ml of DMEM containing 10% rat serum which had been collected from LPS-treated rats. After incubation for 24 h, the cultures were rinsed with methionine-free Eagle's minimum essential medium (MEM), and 0.5 ml of methionine-free MEM supplemented with [³⁵S]methionine (3.7 MBq/ml) was added. After incubation for 6 h, the medium was harvested and centrifuged at 8000 × *g*. The medium (50 μl) was diluted 10 times with immunoprecipitation buffer (I-buffer; 20 mM Tris-HCl (pH 7.4), containing 5 mM EDTA, 0.1 M NaCl and 1% Triton X-100) and mixed with rabbit anti-rat angiotensinogen serum (0.5 μl). After incubation at 4°C overnight, 10% suspension of Staphylosorb (Merck Co., Tokyo, Japan) in I-buffer was added, and the mixture was kept in ice for 30 min. After centrifugation at 8000 × *g* for 5 min, the pellet was washed five times with 1 ml of I-buffer each time and then suspended in 50 μl of 0.125 M Tris-HCl (pH 6.8), 4% SDS, 10% mercaptoethanol, 20% glycerol and 0.2% bromophenol blue. Following heating at 90°C for 2 min, the suspension was centrifuged at 8000 × *g* for 2 min, and then the supernatant (20 μl) was loaded onto a 10% polyacrylamide gel containing 0.1% SDS and subjected to electrophoresis.²⁰⁾ The gel was then fluorographed by treatment with sodium salicylate,²¹⁾ dried and autoradiographed at -90°C.

Results

Development of Tolerance to LPS Induction of Angiotensinogen Animals received a daily dose of LPS (50 μg) or vehicle for 5 consecutive days from day 0 to 4. A primary injection of LPS resulted in about 2-fold elevation of serum angiotensinogen at 24 h (Fig. 1), as reported previously.³⁾ However, subsequent LPS injections on days 1–4 did not result in the appearance of additional serum angiotensinogen peaks, suggesting an LPS-tolerant state. When these rats were again treated with LPS on day 15, no increase in serum angiotensinogen was observed (Fig. 1). In contrast, LPS injection into the vehicle-pretreated rats caused a significant elevation of serum angiotensinogen with a peak at 16 h.

To determine whether the absence of serum angiotensinogen response following repeated LPS stimuli is a reflection of the hyporesponsiveness of angiotensinogen synthesis in the liver, hepatic levels of angiotensinogen mRNA were determined by Northern blot analysis in rats treated with LPS on day 15. The electrophoretic migration of

Fig. 1. Serum Levels of Angiotensinogen in Rats Following Induction of LPS Tolerance

Rats (six per group) were injected with saline (○) or 50 μg of LPS (●) i.p. once a day for 5 consecutive days from day 0 to 4, and then blood samples were collected on days 1–5. On day 15 (arrow), animals which had received either saline or LPS were injected i.p. with 50 μg of LPS, and blood samples were collected at 8, 16, 24, 48 and 96 h. Sera were assayed for angiotensinogen, and results are expressed as the means ± S.D. of 6 animals.

Fig. 2. Northern Blot Analysis of Angiotensinogen mRNA in the Liver of LPS-Treated Rats

Vehicle- or LPS-pretreated rats were injected with LPS on day 15, as described in the legend of Fig. 1. Liver samples were isolated at designated intervals shown in each lane. RNA was extracted and electrophoretically separated on 1.2% agarose gel. After transfer to nylon membrane, RNA was hybridized to the ³²P-labeled angiotensinogen cDNA probe (upper panels) and rehybridized with an oligonucleotide recognizing the 18S rRNA (lower panels). Hybridization was performed as described in Materials and Methods. Representative results of 5 experiments are shown.

angiotensinogen mRNA was close to the position of 18S ribosomal RNA as reported previously.¹⁶⁾ The levels of liver angiotensinogen mRNA in the vehicle-pretreated rats increased 8 h after injection of LPS, whereas no increase was detected in the LPS-pretreated rats, representing an LPS-tolerant state in liver angiotensinogen synthesis (Fig. 2). These differences cannot be attributed to the variation in the amounts of total RNA in individual samples applied to the gels, due to the fact that the hybridization with an oligonucleotide that recognizes rat 18S rRNA showed a similar amount of 18S rRNA among the samples.

Development of Tolerance to LPS Induction of α_2 -Macroglobulin α_2 -Macroglobulin is one of the acute-phase

proteins in the rat, and its production in the liver is regulated by IL-6 under inflammatory conditions.²²⁾ As shown in Fig. 3, a marked rise in serum α_2 -macroglobulin was observed in rats by a repeated injection of LPS; the levels reached a peak on day 2 and then began to decrease regardless of subsequent injections of LPS. Administration of LPS to the vehicle-pretreated rats on day 15 caused a marked rise in serum α_2 -macroglobulin, whereas no increase was observed in LPS-pretreated rats (Fig. 3), indicating an LPS-tolerant state in the response of serum α_2 -macroglobulin.

Development of Tolerance to LPS Induction of Serum Factor Responsible for the Enhancement of Angiotensinogen Synthesis in Hepatoma Cells The ability of sera to increase angiotensinogen synthesis by hepatoma H4 cells was evaluated *in vitro* to determine whether the absence of angiotensinogen response in LPS-tolerant rats resulted from a lack of certain factors inducing liver angiotensinogen synthesis. On day 15 after vehicle or LPS-treatment, serum samples were collected following injection of LPS and then added to the culture medium of H4 cells at 10%

concentration. Cells were cultured for 24 h in the presence of serum samples and then for an additional 6 h in the presence of [³⁵S]methionine. Radiolabeled angiotensinogen newly synthesized by H4 cells was immunoprecipitated by anti-rat angiotensinogen and analyzed by SDS-PAGE. As shown in Fig. 4, the immunoprecipitate contained a radioactive band with an apparent molecular mass of about 58000, which comigrated with ¹²⁵I-labeled rat angiotensinogen. Serum samples that had been collected from the vehicle-pretreated rats 2 and 4 h after injection of LPS showed a stimulation of angiotensinogen synthesis by H4 cells, as compared to sera collected from non-treated rats (0 h). On the other hand, no such stimulatory activity on angiotensinogen synthesis was observed in sera collected from LPS-pretreated rats after the secondary LPS exposure. These results indicate that a factor responsible for LPS-induced enhancement of angiotensinogen synthesis appears in serum 2–4 h after a primary injection of LPS,

Fig. 3. Serum Levels of α_2 -Macroglobulin in Rats Following Induction of LPS Tolerance

Rats (six per group) were pretreated with saline (○) or LPS (●) for 5 d and then injected with LPS on day 15, as described in the legend of Fig. 1. Sera were assayed for α_2 -macroglobulin, and results are expressed as the means $\pm$ S.D. of 4 animals.

Fig. 4. Enhancement of Angiotensinogen Synthesis in Rat Hepatoma H4 Cells by Sera Collected from LPS-Treated Rats

Vehicle- or LPS-pretreated rats were injected with LPS on day 15, as described in the legend of Fig. 1. Sera were collected after injection of LPS at the designated intervals shown and added to culture medium of H4 cells at 10% concentration. Following culture for 18 h, cells were labeled with [³⁵S]methionine for 6 h, and the medium was then harvested. An aliquot of the medium was immunoprecipitated with antibody against rat angiotensinogen, and the radiolabeled immunoprecipitate was analyzed by SDS-polyacrylamide gel (10%) electrophoresis. Number shown in each lane indicates hours after LPS injection. Std represents an authentic ¹²⁵I-labeled rat angiotensinogen. Representative results of 4 experiments are shown.

Fig. 5. Serum Levels of TNF and IL-6 Following Induction of LPS Tolerance

Vehicle- (○) or LPS-pretreated (●) rats were injected with LPS on day 15, as described in the legend of Fig. 1. Sera were collected after injection of LPS at designated intervals and assayed for TNF and IL-6 by bioassays using L929 and MH60 cells, respectively. Results are expressed as the means $\pm$ S.E. of 5 animals.

and that the LPS-induced appearance of this factor is refractory to a secondary LPS stimulus in the LPS-tolerant state.

Development of Tolerance to LPS Induction of TNF and IL-6 Serum levels of TNF and IL-6 were measured in LPS-tolerant and non-tolerant rats after injection of LPS on day 15 to evaluate the role of these cytokines in LPS-induced synthesis of angiotensinogen. As shown in Fig. 5, the injection of LPS into the vehicle-pretreated rats resulted in a rapid rise in serum TNF with a peak at 2 h. The peak was transitory, and no TNF was detected at 4 h. The kinetics of the increase in serum IL-6 were delayed for TNF with a peak at 4 h (Fig. 5). In LPS-tolerant rats, however, no increases in serum TNF or IL-6 were observed, indicating that the LPS-tolerant state was also developed in serum levels of TNF and IL-6, as well documented by other investigators.⁹⁻¹¹⁾

Discussion

The present studies have demonstrated that hyporesponsiveness of LPS-induced elevation of plasma angiotensinogen can be induced by repetitive LPS exposure. It seems likely that this hyporesponsiveness is primarily due to reduced response of liver angiotensinogen synthesis to LPS stimulus, because the increase of angiotensinogen mRNA was not observed in the liver. Sera collected from LPS-treated rats were found to stimulate angiotensinogen synthesis in cultured hepatoma cells, suggesting the presence of serum factor that mediates between LPS stimulus and angiotensinogen synthesis in the liver. Hyporesponsiveness by a repetitive LPS exposure was also observed in LPS-induced formation of the serum factor. Thus it is likely that a failure of LPS to produce the factor results in the hyporesponsiveness of LPS-induced synthesis of angiotensinogen.

The mechanisms involved in the establishment of LPS tolerance remain poorly defined. However, a series of recent studies has suggested that the phenomenon of LPS tolerance involves down-regulation of the production of cytokines like TNF, IL-1, IL-6 and colony-stimulating factor.^{9-11,22)} Consistent with these studies, reduced responses of serum TNF and IL-6 to an LPS stimulus were observed in rats after repetitive LPS exposure (Fig. 5). Thus it is reasonable to assume that, in a state of LPS tolerance, there is a reduced response of acute-phase proteins, whose syntheses are regulated by cytokines. Actually, LPS-induced elevation of serum α_2 -macroglobulin, whose synthesis in the liver is regulated by IL-6,²³⁾ was reduced after repetitive LPS exposure. Thus the hyporesponsiveness of LPS-induced synthesis of angiotensinogen seems ascribable to the reduced response of cytokines in LPS tolerance. The maximum activity of the serum factor stimulating angiotensinogen synthesis in cultured hepatoma cells was observed 4 h after injection of LPS (Fig. 4). It is unlikely that TNF is the factor because of the fact that no TNF was detected in the sera of rats by 4 h. As reported in our previous study,⁸⁾ angiotensinogen synthesis by cultured H4 cells is enhanced by the addition of human recombinant IL-6, but not by IL-1, suggesting that IL-6 is identical to the serum factor. Evidence supporting this hypothesis is that the kinetics of the increase in serum factor after injection of LPS were

similar to those of IL-6 with a peak at 4 h. In addition, LPS-induced increase in serum IL-6 was absent in LPS-tolerant rats, supporting that the hyporesponsiveness of LPS-induced synthesis of angiotensinogen results from a failure of IL-6 formation in LPS tolerance. It therefore seems likely that LPS-induced increase in angiotensinogen synthesis is mediated by IL-6.

IL-6 has recently been recognized as a major mediator of the hepatic synthesis of acute-phase proteins,^{23,24)} indicating that its action is not restricted to the immune system. The importance of the IL-6-regulated angiotensinogen expression in infectious diseases with gram-negative bacteria remains to be determined. However, many investigators have provided evidence supporting that the plasma level of angiotensinogen is one of the rate-limiting factors in the generation of angiotensin I²⁵⁾ and ultimately important in the regulation of blood pressure. Thus, the observations in this study may also be further indicative of communication between the immune and endocrine systems.

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