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INTERLEUKIN-6 AS A MEDIATOR RESPONSIBLE FOR INFLAMMATION-INDUCED INCREASE IN PLASMA ANGIOTENSINOGEN

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Abstract—The concentration of plasma angiotensinogen increases upon induction of inflammation. Studies were carried out using serum samples collected from mice and rats after injection of lipopolysaccharide (LPS) to determine whether interleukin-6 (IL-6) is a mediator responsible for the inflammation-induced increase of angiotensinogen synthesis in liver cells. Serum collected from mice or rats 2 and 4 hr after injection of LPS contained a factor that stimulated [^{35}S]methionine incorporation into angiotensinogen newly synthesized by rat hepatoma H4IIEC3 (H4) cells. Assay of IL-6 using an IL-6-dependent murine hybridoma, MH60.BSF2 cells, showed the presence of IL-6-like activity in sera of mice or rats 2 and 4 hr after injection of LPS. Anti-mouse IL-6 monoclonal antibody completely inhibited not only the IL-6-like activity present in LPS-treated mouse serum but also the ability of the serum to stimulate angiotensinogen synthesis of H4 cells. These results suggest that increased synthesis of angiotensinogen in the liver after induction of inflammation is mediated by IL-6, a cytokine important in immune reactions and the hepatic acute-phase response.

Angiotensinogen, a plasma protein synthesized mainly by the liver, is cleaved by renin to release a decapeptide, angiotensin I. Subsequently, angiotensin I is further hydrolyzed by converting enzyme to produce the octapeptide, angiotensin II, a biologically active molecule that plays an important role in the regulation of blood pressure and electrolyte balance [1]. Many investigators have provided evidence that, in addition to the concentration of renin in plasma, the plasma concentration of angiotensinogen is a rate-limiting factor in the generation of angiotensin I [1]. Although angiotensinogen mRNA has been identified in several tissues [2, 3], most of the angiotensinogen present in plasma is derived from the liver [1].

Acute inflammation induced by lipopolysaccharide (LPS)† causes an elevation of liver angiotensinogen mRNA [4], as well as the plasma concentration of angiotensinogen [5]. Our previous studies have shown that the LPS-induced elevation of plasma angiotensinogen is mediated by a leukocyte-derived factor [6]. Recently we found that human recombinant interleukin-6 (IL-6) was able to stimulate angiotensinogen production in rat hepatoma H35 cells [7] and in primary cultures of rat hepatocytes [8] *in vitro*, suggesting that this cytokine is a possible mediator responsible for the LPS-induced increase in angiotensinogen synthesis in the liver. However, no further evidence supporting this hypothesis has been reported.

In the present study, we found that serum collected from LPS-treated mice or rats contained a factor that stimulated angiotensinogen synthesis by rat hepatoma H4IIEC3 (H4) cells. The results indicated that this factor is identical to IL-6.

MATERIALS AND METHODS

Materials. Human recombinant IL-6 (5.2×10^6 U/mg) and an IL-6-dependent murine hybridoma clone, MH60.BSF2 cells, were supplied by Dr. T. Hirano (Biomedical Research Center, Osaka University Medical School, Osaka, Japan). Reuber rat hepatoma H4 cells were purchased from the Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). The following chemicals were obtained from commercial sources: Dulbecco's modified Eagle's medium (DMEM), Eagle's minimum essential medium (MEM), methionine-deficient MEM and RPMI-1640 medium from the Nissui Pharmaceutical Co. (Tokyo, Japan); [^{35}S]methionine from New England Nuclear (Boston, MA, U.S.A.); LPS (*Staphylococcus typhosa* 0910) from Difco (Detroit, MI, U.S.A.); 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and phenylmethylsulfonyl fluoride (PMSF) from Nacalai Tesque (Kyoto, Japan); Soluen-350 from Packard Instruments, Inc. (Downers Grove, IL, U.S.A.); and Staphylosorb from the Mercian Co. (Tokyo, Japan). Rat anti-mouse IL-6 monoclonal antibody was obtained from the Genzyme Co. (Cambridge, MA, U.S.A.). This antibody has no cross-reactivity with mouse tumor necrosis factor-α, granulocyte-

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† Abbreviations: LPS, lipopolysaccharide; IL-6, interleukin-6; DMEM, Dulbecco's modified Eagle's medium; MEM, Eagle's minimum essential medium; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PMSF, phenylmethylsulfonyl fluoride; FBS, fetal bovine serum; and SDS, sodium dodecyl sulfate.

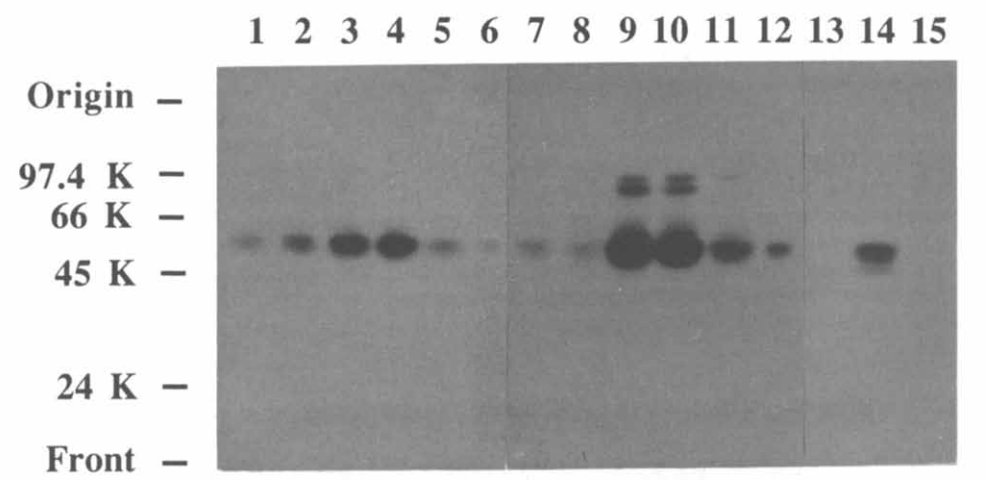

Fig. 1. Stimulation of angiotensinogen synthesis in rat hepatoma H4IIEC3 (H4) cells by serum collected from LPS-treated mouse or rat. Hepatoma cells were incubated with mouse serum (lanes 1–5 and 13–15) or rat serum (lanes 7–11) at a 10% concentration for 18 hr. Lanes represent sera collected from untreated animals (lanes 1 and 7), 1 hr (lanes 2 and 8), 2 hr (lanes 3, 9 and 13–15), 4 hr (lanes 4 and 10) and 8 hr (lanes 5 and 11) after LPS injection. Lanes 6 and 12 represent ^{125}I-labeled rat angiotensinogen. The cultures were labeled with [^{35}S]methionine for 6 hr, and the medium was then harvested. An aliquot of the medium was reacted with antibody against rat angiotensinogen, except for lane 13, and the radiolabeled immunoprecipitate was analyzed by SDS–polyacrylamide gel (10%) electrophoresis (SDS–PAGE). The immunoprecipitation at lane 13 was carried out by rabbit preimmune serum, and at lane 15 in the presence of purified rat angiotensinogen (50 μg/ml). The results shown are from one of three representative experiments.

monocyte colony-stimulating factor, or human and rat IL-6, according to the product specifications by the Genzyme Co. Purification of rat angiotensinogen and preparation of rabbit anti-rat angiotensinogen were described previously [7].

Animals and treatment. Male Sprague–Dawley rats (120–150 g) and male ICR mice (30–35 g) were purchased from the Shizuoka Laboratory Animal Center (Hamamatsu, Japan). Acute inflammation was induced by a single intraperitoneal injection of LPS dissolved in saline at a dose of 50 μg/100 g body weight. At different times after LPS injection, blood was collected from the abdominal aorta under pentobarbital anesthesia, and the serum was prepared.

Assay of IL-6. IL-6 was measured by bioassay using an IL-6-dependent murine hybridoma clone, MH60.BSF2 cells [9]. Cells (1×10^4) were cultured in a 96-well microplate with 200 μL of RPMI-1640

Table 1. Serum interleukin-6 activity in mouse and rat after injection of lipopolysaccharide

Time after LPS injection (hr)	Serum interleukin-6 level (U/mL)	
	Mouse	Rat
0	<0.005	<0.005
1	34.4 ± 4.9*	<0.005
2	858.0 ± 62.4*	2736.0 ± 1094.0*
4	429.0 ± 71.7*	888.0 ± 192.3*
8	13.5 ± 7.9	20.8 ± 6.3*
24	0.5 ± 0.2	0.1 ± 0.0

Values are the means ± SEM of serum samples from four animals. The statistical significance of the differences was analyzed by the Mann–Whitney *U* test.
* Significantly different from 0 hr ($P < 0.05$).

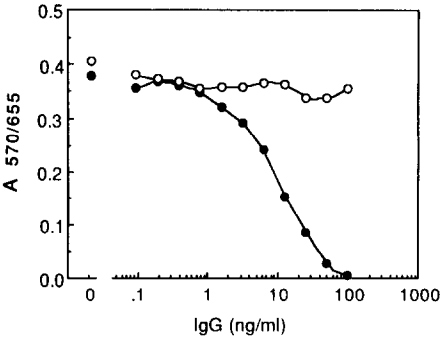

Fig. 2. Effect of anti-mouse interleukin-6 (IL-6) antibody on the proliferation of MH60.BSF2 cells induced by serum collected from an LPS-treated mouse. MH60.BSF2 cells were incubated with 200 μL of medium containing mouse serum (0.15%), which had been collected from a mouse 2 hr after LPS injection, in the presence of rat anti-mouse IL-6 antibody (●) or rat preimmune IgG (○). Abscissa: immunoglobulin concentration in cultures; ordinate: formazan formation by cells, measured at 570 nm (test wavelength) and 655 nm (reference wavelength). Values are expressed as means of duplicate assays.

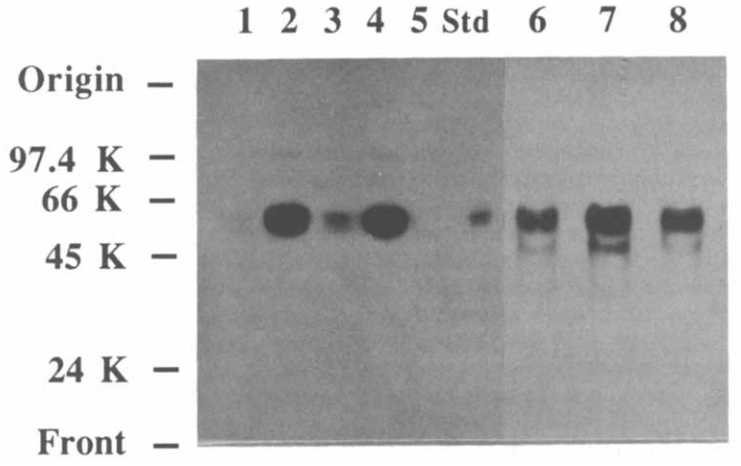

Fig. 3. Effect of anti-mouse IL-6 antibody on the activity stimulating angiotensinogen synthesis by H4 cells in serum collected from an LPS-treated mouse. Hepatoma cells were incubated with fetal bovine serum, untreated mouse serum or LPS-treated mouse serum at a 10% concentration in the presence or absence of anti-mouse IL-6 antibody or preimmune immunoglobulin for 18 hr. LPS-treated mouse serum was collected 2 or 4 hr after LPS injection. The cultures were labeled with [^{35}S]methionine for 6 hr, and the radiolabeled immunoprecipitate of the medium against angiotensinogen antibody was analyzed by SDS–PAGE (see legend to Fig. 1). Lanes 1–5 show immunoprecipitate of the medium of H4 cells, which were treated with fetal bovine serum alone (lane 1), LPS-treated 2-hr mouse serum alone (lane 2), LPS-treated 2-hr mouse serum plus anti-mouse IL-6 antibody (50 μg/mL) (lane 3), LPS-treated 2-hr mouse serum plus rat preimmune IgG (50 μg/mL) (lane 4), untreated mouse serum alone (lane 5), or ^{125}I-labeled rat angiotensinogen (Std). Lanes 6–8 also show immunoprecipitate of the medium of H4 cells, which were treated with fetal bovine serum alone (lane 6), LPS-treated 4-hr mouse serum alone (lane 7) or LPS-treated 4-hr mouse serum plus anti-mouse IL-6 antibody (50 μg/mL) (lane 8). The results shown are from one of four representative experiments.

medium containing 10% fetal bovine serum (FBS) and various concentrations of recombinant human IL-6 or serum samples for 72 hr. Then the amount of formazan generated from MTT by the cells was measured by a microplate reader (Bio-Rad model 3550) using a test wavelength of 570 nm and a reference wavelength of 655 nm [10]. IL-6 in serum samples was estimated as the amount equivalent to recombinant human IL-6.

Radiolabeling of hepatoma cell cultures. H4 cells were grown on 24-well culture plates in DMEM, supplemented with 5% FBS, at 37° in 95% air and 5% CO_2. After reaching confluence, the cells were washed with FBS-free DMEM, and the medium was replaced with 0.5 mL of DMEM supplemented with 10% serum collected from mice or rats. After incubation for 18 hr, the cultures were rinsed three times with methionine-deficient MEM, and 0.5 mL of methionine-deficient MEM supplemented with [^{35}S]methionine (100 μCi/mL) was added. The cultures were incubated for an additional 6 hr, and the medium was then harvested and subjected to centrifugation at 8000 *g*. One part of 0.2 M Tris–HCl buffer, pH 7.4, containing 1 M NaCl, 50 mM disodium EDTA, 10% Triton X-100, 1 mM PMSF, leupeptin (10 μg/mL) and 1% bovine serum albumin was added to nine parts of labeled supernatant.

Immunoprecipitation and polyacrylamide gel analysis. An aliquot of the treated labeled culture medium (50 μL) was mixed with 0.5 mL of immunoprecipitation buffer (I-buffer; 20 mM Tris–HCl, pH 7.4, containing 5 mM disodium EDTA, 0.1 M NaCl and 1% Triton X-100) and 0.5 μL of rabbit anti-rat angiotensinogen serum. After incubation at 4° for 18 hr, 50 μL of 10% suspension of Staphylosorb in I-buffer was added, followed by incubation for an additional 30 min at 4°. The mixture was subjected to centrifugation at 8000 *g* for 5 min, the supernatant was decanted, and the pellet was washed five times with 1 mL of I-buffer each time. The pellet was resuspended in 50 μL of 0.125 M Tris–HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, 20% glycerol and 0.2% bromophenol blue, heated at 90° for 2 min, and then subjected to centrifugation at 8,000 *g* for 2 min. The supernatant (20 μL) was loaded onto a 10% polyacrylamide gel containing 0.1% SDS and subjected to electrophoresis [11]. The gel was then fluorographed by treatment with sodium salicylate [12], dried and autoradiographed at −90°.

Statistical analysis. Data are expressed as means ± SEM. Statistical analysis was performed by Student's *t*-test or by the Mann–Whitney *U* test [13]. The level of statistical significance was set at $P < 0.05$.

RESULTS

Stimulation of angiotensinogen synthesis in H4 cells by LPS-treated mouse or rat serum. The synthesis of angiotensinogen by H4 cells was investigated by direct immunoprecipitation. The cells were cultured for 18 hr in the presence of 10%

normal mouse or rat serum, washed to remove the serum, and then incubated with [^{35}S]methionine for 6 hr. Then the medium was immunoprecipitated with anti-angiotensinogen antibody and analyzed by SDS–polyacrylamide gel electrophoresis. As shown in Fig. 1, the immunoprecipitate of H4-cell conditioned medium contained a radioactive band with an apparent molecular mass of about 58,000 (lanes 1 and 7), which could not be distinguished from the radioactive band of ^{125}I-labeled rat angiotensinogen (lanes 6 and 12).

We then investigated whether angiotensinogen synthesized by the metabolically labeled cells was influenced by pretreatment with mouse or rat serum, which had been collected from LPS-treated animals. H4 cells were precultured with 10% LPS-treated animal serum for 18 hr and then cultured for an additional 6 hr in the presence of [^{35}S]methionine. As shown in Fig. 1, mouse sera that had been collected 2 and 4 hr (lanes 3 and 4, respectively) after injection of LPS stimulated the synthesis of angiotensinogen by H4 cells, as compared with cells cultured with normal mouse serum (lane 1). Mouse sera collected 1 and 8 hr after injection of LPS did not exhibit any stimulatory effect on angiotensinogen synthesis (lanes 2 and 5, respectively). To confirm that the radioactive band with 58,000 was angiotensinogen, the conditioned medium of H4 cells was prepared by treating cells with mouse serum, which had been collected 2 hr after injection of LPS, and then immunoprecipitated with rabbit preimmune serum instead of rabbit anti-angiotensinogen serum or in the presence of excess unlabeled angiotensinogen. As shown in Fig. 1, the radioactive band with 58,000 (lane 14) was not observed when the immunoprecipitation was carried out with rabbit preimmune serum (lane 13), or when excess unlabeled angiotensinogen was present (lane 15). These results indicated that the 58,000 protein specifically immunoprecipitated by the anti-angiotensinogen antibody was angiotensinogen newly synthesized by H4 cells, and its synthesis by H4 cells was stimulated by LPS-treated mouse serum.

Similar results were obtained with LPS-treated rat sera (lanes 7–11 in Fig. 1), but the stimulatory activity was more pronounced than that of LPS-treated mouse serum. Additional labeled bands with higher molecular weight than angiotensinogen were observed in immunoprecipitates in culture medium of rat sera-treated cells (lanes 9–11), but they were not competed when the immunoprecipitation was carried out in the presence of excess unlabeled angiotensinogen (data not shown).

Serum IL-6 activity in mouse or rat after injection of LPS. We reported previously that recombinant human IL-6 was able to stimulate angiotensinogen production by rat hepatoma H35 cells [7] and rat hepatocytes in primary culture [8]. To determine whether the stimulatory activity found in LPS-treated mice or rats on angiotensinogen synthesis by H4 cells is associated with endogenous IL-6, the activity of IL-6 in serum was measured after injection of LPS by bioassay using murine hybridoma clone, MH60.BSF2 cells. As shown in Table 1, serum IL-6 activity was increased 2 hr after injection of LPS in either animal. High activity of IL-6 was still observed at 4 hr in both animals, but decreased to lower levels at 8 hr. The elevation of serum IL-6 activity in response to LPS injection was much more pronounced in rats than in mice. These profiles of serum IL-6 activity in the two animal species were consistent with those of the stimulatory activity on angiotensinogen synthesis by H4 cells.

Serum IL-6 activity was determined by bioassay, which measured IL-6-dependent proliferation of murine hybridoma cells. To confirm that the IL-6-like activity present in serum of LPS-treated animals affecting the growth of MH60.BSF cells was, in fact, due to the action of IL-6, the effect on this assay of rat anti-mouse IL-6 monoclonal antibody was examined. As shown in Fig. 2, the IL-6 activity present in mouse serum, which had been collected 2 hr after injection of LPS, was neutralized by rat anti-mouse IL-6 antibody, but not by rat preimmune IgG. This result indicated that IL-6 activity measured in this study truly reflected the IL-6 concentration present in serum, at least in mice.

Effect of anti-mouse IL-6 antibody on the activity present in serum of LPS-treated mice stimulating angiotensinogen synthesis. Since anti-mouse IL-6 antibody was able to neutralize IL-6 activity present in LPS-treated mouse serum, it was considered of interest to determine whether the antibody influenced

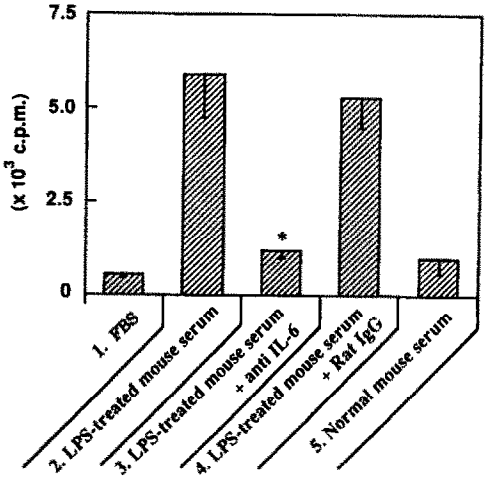

Fig. 4. Inhibition of angiotensinogen synthesis in H4 cells by anti-mouse IL-6 antibody. Following SDS–PAGE of immunoprecipitate as described in Fig. 1, the radioactive band corresponding to angiotensinogen was cut, dissolved in Soluen-350 and the radioactivity counted in a scintillation counter. Experiments were carried out on serum samples, from four untreated mice and four LPS-treated mice, that had been collected 2 hr after LPS injection. Columns show radioactivities of metabolically labeled angiotensinogen by H4 cells in the presence of fetal bovine serum alone (column 1), LPS-treated mouse serum alone (column 2), LPS-treated mouse serum plus anti-mouse IL-6 antibody (50 μg/mL) (column 3), LPS-treated mouse serum plus rat preimmune IgG (column 4) and untreated mice sera alone (column 5). Values are means ± SEM of serum samples from four mice, and the statistical significance of the differences was analyzed by Student's *t*-test. Key: (*) significantly different from column 2 or 4 ($P < 0.001$), but not from column 5 ($P > 0.05$).

the stimulatory activity of serum from LPS-treated mice on angiotensinogen synthesis. As shown in Fig. 3, addition of mouse serum, which had been collected 2 hr after injection of LPS, to culture medium at a 10% concentration stimulated angiotensinogen synthesis by H4 cells (lane 2), whereas normal mouse serum did not (lane 5). This stimulatory activity was abolished when rat anti-mouse IL-6 antibody was added to the culture medium at a concentration of 50 μg/mL (lane 3), but not in the presence of rat preimmune IgG (lane 4). Similarly the angiotensinogen synthesis by H4 cells was stimulated by mouse serum collected 4 hr after injection of LPS (lane 7) in comparison with that from normal mouse (lane 6), and its stimulatory activity was blocked by the presence of anti-IL-6 antibody (lane 8).

When radioactive bands corresponding to authentic angiotensinogen were cut from the gels, dissolved in Soluen-350 and their radioactivity counted in a scintillation counter, it was confirmed that the stimulatory activity present in mouse serum collected 2 hr after injection of LPS was blocked almost completely by the anti-IL-6 antibody (Fig. 4). Anti-IL-6 antibody alone affected neither the viability of H4 cells nor their angiotensinogen synthesis. These results indicated that the activity stimulating angiotensinogen synthesis by H4 cells present in LPS-treated mouse serum was due exclusively to an increased concentration of IL-6.

DISCUSSION

In the present study, we found that serum collected from LPS-treated mice or rats had the ability to stimulate angiotensinogen synthesis by rat hepatoma H4 cells. The stimulatory activity on angiotensinogen synthesis was found in sera collected 2 and 4 hr after injection of LPS, and these sera also contained high levels of IL-6 activity. Furthermore, the stimulatory activity on angiotensinogen synthesis, as well as IL-6 activity, in mouse serum was blocked by anti-mouse IL-6 antibody. Since IL-6 levels in mouse or rat serum were estimated to be more than 400 or 800 U/mL, respectively, at 2 and 4 hr after injection of LPS, the culture media of H4 cells supplemented with a 10% concentration of these sera seem to contain more than 40 U/mL of IL-6. As reported previously [7], angiotensinogen production by rat hepatoma cells was stimulated by human recombinant IL-6, and the maximum response was obtained at 50 U/mL, suggesting that the addition of these sera at a 10% concentration into culture medium produced a maximum response of H4 cells. In fact, there was no difference in the stimulatory activity on angiotensinogen synthesis between sera collected 2 and 4 hr after injection of LPS, whereas higher levels of IL-6 in 2-hr serum than those in 4-hr serum were found in both animals (Fig. 1).

We were unable to confirm whether a serum factor present in LPS-treated rats was also identical to rat IL-6, because anti-rat IL-6 antibody is not yet commercially available. However, the fact that the stimulatory activity in LPS-treated rat serum was closely correlated with serum IL-6 concentration lends support to the possibility that the stimulatory activity was due to the action of rat IL-6. Thus, it is reasonable to conclude that IL-6 is a mediator responsible for the inflammation-induced increase of angiotensinogen synthesis in the liver.

Angiotensinogen has been identified as a member of the serine-proteinase inhibitor (serpin) superfamily [14], though like ovalbumin, angiotensinogen has no known proteinase inhibitory capacity [15]. Most of the serpin superfamily, such as α_1-antitrypsin, α_1-antichymotrypsin and α_2-antiplasmin, are also known to be acute-phase proteins, characterized by increased synthesis in the liver in response to tissue injury caused by infection or trauma [16]. Furthermore, it has been demonstrated recently that IL-6 is a major mediator in the hepatic acute phase response [17]. The level of angiotensinogen mRNA in the liver [4], as well as the concentration in plasma [5, 6], is increased in the rat after LPS treatment. Elevation of plasma angiotensinogen has also been demonstrated in humans during infection [18]. Thus, it is likely that angiotensinogen is not only a member of the serpin superfamily but also a plasma protein belonging to the acute-phase proteins. In this context, two groups of investigators have reported that angiotensinogen is not a typical acute-phase protein [19, 20]. Both groups observed that acute inflammation induced by turpentine injection did not result in an increase of angiotensinogen concentration in rat plasma, and therefore suggested that the LPS-induced increase of angiotensinogen synthesis in the liver following LPS injection is mediated by a mechanism different from that of other acute-phase proteins. However, as demonstrated in this study, it seems unlikely that increased synthesis of angiotensinogen in the liver by LPS treatment is mediated by some factor(s) besides IL-6. In addition, we have observed that turpentine injection also caused a slight but significant increase of plasma angiotensinogen in the rat (unpublished data). Thus, it seems reasonable to conclude that angiotensinogen is one of the acute-phase proteins regulated by IL-6, and thus behaves as a typical acute-phase protein.

Increases in the plasma concentration of angiotensinogen would be expected to increase the rate of formation of angiotensin I, since the plasma concentration of angiotensinogen is much lower than the concentration required to produce the maximum velocity of the renin-angiotensinogen reaction [1]. This appears to be the case in LPS-treated rats in which both plasma renin activity and the plasma concentration of angiotensinogen are elevated when the plasma renin concentration is within the normal range [21]. In addition to the system generating angiotensin II involving renin and converting enzyme, a neutrophil neutral proteinase, cathepsin G, has been demonstrated to generate angiotensin II directly from angiotensinogen [22]. Since the early response in inflammatory reactions involves migration of granulocytes and monocytes and release of cell constituents including cathepsin G [23], it is possible that the inflammation-induced increase of plasma angiotensinogen contributes to local generation of angiotensin II, which is a potent vasoconstrictor and an inducer of platelet-activating factor from endothelial cells [24]. However, the precise functional roles of angiotensinogen following injury and infection remain to be investigated.

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