ANG II is involved in the LPS-induced production of proinflammatory cytokines in dehydrated rats

Michio Miyoshi, Katsumi Nagata, Toshiaki Imoto, Osamu Goto, Akiko Ishida, and Tatsuo Watanabe

The Department of Physiology, Tottori University Faculty of Medicine, Yonago, Tottori 683, Japan

Submitted 8 November 2002; accepted in final form 19 December 2002

ANG II is involved in the LPS-induced production of proinflammatory cytokines in dehydrated rats. Am J Physiol Regul Integr Comp Physiol 284: R1092–R1097, 2003; 10.1152/ajpregu.00700.2002.—We have previously reported results that led us to speculate that ANG II is involved in the LPS-induced production of proinflammatory cytokines, especially under dehydrated conditions. To test this possibility, in this study we examined the effects of an angiotensin-converting enzyme (ACE) inhibitor and an antagonist of the type-1 ANG II receptor (AT1 receptor) on the LPS-induced production of the proinflammatory cytokines IL-1 and IL-6 in dehydrated rats. A single intravenous injection of LPS induced a marked increase in the expression of IL-1β mRNA in the liver, an effect that was significantly attenuated by pretreatment with the ACE inhibitor. Furthermore, the ACE inhibitor reduced the LPS-induced increase in the hepatic concentration of IL-1β protein. When the AT1 receptor antagonist was given intravenously before the LPS, the increase in the hepatic concentration of IL-1β was significantly reduced. Finally, the ACE inhibitor reduced the LPS-induced increase in the plasma concentration of IL-6. These results represent the first in vivo evidence that ANG II and its AT1 receptor play important roles in the production of proinflammatory cytokines that is induced by LPS under dehydrated conditions.

PROINFLAMMATORY CYTOKINES such as IL-1 or IL-6 are members of a family of endogenous pyrogens (EP), production of which is powerfully stimulated by LPS (6). Occasionally, we experience a high fever in bacteria-infected patients under dehydrated conditions. In 1986, Morimoto et al. (14) showed that intravenous injection of LPS induced a fever that was significantly greater in dehydrated rats than in euvacuolated rats. However, dehydration had no effect on the fever induced by intravenous injection of their “homemade” crude EP. Accordingly, the fever enhancement caused by dehydration may be due to increased production of EP in response to LPS. Because the secretion of ANG II increases under dehydrated conditions, we recently tested the possibility that ANG II is involved in this fever enhancement. In fact, the LPS-induced fever seen by us in dehydrated rats was significantly attenuated by an angiotensin-converting enzyme (ACE) inhibitor given intravenously, whereas the IL-1-induced fever underwent no alterations with this inhibitor (27). Taken together, the above evidence makes it likely that ANG II contributes to the LPS-induced production of EP, or of a proinflammatory cytokine such as IL-1, and that this leads to an enhancement of the LPS-induced fever seen under dehydrated conditions.

Recent evidence suggests that ANG II may itself be a proinflammatory peptide. For example, ANG II induces an inflammatory response, involving increases in the expressions of such proinflammatory enzymes as phospholipase (23) and NAD(P)H oxidase (4), and type-1 ANG II receptors (AT1 receptors) are involved in certain types of cardiovascular inflammation (26). Furthermore, ACE inhibitors have an anti-inflammatory effect (2, 13, 19). These findings support the above-mentioned possibility that ANG II participates in the LPS-induced production of proinflammatory cytokines by acting as a proinflammatory peptide.

The present study was carried out to investigate whether the LPS-induced production of proinflammatory cytokines does indeed involve mediation by ANG II. We examined the effect produced by an intravenous injection of either an ACE inhibitor or an AT1-receptor antagonist on the LPS-induced production of proinflammatory cytokines, such as IL-1 or IL-6, in dehydrated rats. The results revealed that the LPS-induced increase in the expression of IL-1β mRNA in the liver, a representative organ of the reticuloendothelial system, was significantly attenuated by pretreatment with an ACE inhibitor, as was the LPS-induced increase in the liver concentration of IL-1β. When an AT1-receptor antagonist was given before the LPS, the increase in the hepatic concentration of IL-1β was significantly reduced. The ACE inhibitor also reduced the LPS-induced increase in the plasma concentration of IL-6. These results suggest that ANG II and its AT1 receptor play important roles in the LPS-induced production of proinflammatory cytokines, certainly under dehydrated conditions.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: T. Watanabe, Dept. of Physiology, Tottori Univ. Faculty of Medicine, Yonago Tottori 683, Japan (E-mail: watanabe@grape.med.tottori-u.ac.jp).
MATERIALS AND METHODS

Animals

The animals used in this study were male Wistar rats, weighing 270–350 g. They were housed in individual plastic cages (40 × 25 × 25 cm; length × width × depth) with wood-chip bedding in a room maintained at 26 ± 1°C, a temperature within the thermoneutral zone for rats. They experienced a 12:12-h light-dark photoperiod, lights coming on at 0700. All animals had ad libitum access to drink and standard laboratory rat chow. The protocols were reviewed by the Committee on the Ethics of Animal Experiments in Tottori University Faculty of Medicine, and the experiments were carried out in accordance with the Guidelines for Animal Experiments at Tottori University Faculty of Medicine and with the Federal Law (No. 221) and Notification (No. 6) of the Japanese Government.

This study comprised three types of experiment (see below), all on freely moving rats. All rats were dehydrated by deprivation of drinking water for 24 h before experimentation. Rats lost ~6% of their total body weight as a result of this deprivation. In our previous study (27), the LPS-induced fever was significantly attenuated by an ACE inhibitor in both dehydrated and euhydrated rats, but the effect was greater in the former. For that reason, we used dehydrated rats in the present study.

In experiment 1, we investigated the effect of an intravenous injection of an ACE inhibitor, lisinopril, on the LPS (2 μg/kg iv)-induced change in the production of IL-1β in the liver, both IL-1β mRNA and IL-1β protein being measured. We administered LPS at a dose of 2 μg/kg iv because in our previous study the fever induced by intravenous injection of LPS at this dose was found to be enhanced by dehydration (14). In experiment 2, a single intravenous injection of an AT1-receptor antagonist, losartan, was given, and its effect on the LPS-induced changes in the production of IL-1β in the liver was examined, the hepatic IL-1β protein content being measured. In experiment 3, blood was taken from dehydrated rats to investigate the effect of losartan on the LPS-induced changes in the plasma concentrations of IL-1β. Actually, IL-1 was has been reported not to be detectable in the plasma even after injection of LPS (6). In contrast, the plasma concentrations of another proinflammatory cytokine, IL-6, is reportedly increased by the systemic injection of LPS (6). For that reason, we decided to investigate the effect of losartan on the LPS-induced change in the plasma level of IL-6, too.

Surgery

For intravenous injections and blood sampling, rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and a polyvinyl tube was inserted into the jugular vein so that its tip lay in the superior caval vein near the right atrium (5). The free end of the catheter was passed subcutaneously to the midscapular region, where it was exteriorized dorsally behind the neck. It was kept patent by flushing it every day with heparinized 0.9% saline (50 U/ml). This implantation was performed at least 3 days before the start of the experiment.

All rats were handled for 15 min each day for at least 5 days to accustom them to the experimenters.

Drugs

The LPS used in this study was derived from Salmonella typhosa endotoxin, and it was dissolved in sterile saline. The LPS used in experiments 1 and 3 (i.e., the lisinopril experiments) was purchased from Difco Laboratories (Detroit, MI). Unfortunately, Difco Laboratories stopped production of LPS before we began experiment 2 (i.e., the losartan experiment). We therefore used LPS obtained from Sigma (St. Louis, MO) for experiment 2. Lisinopril (Sigma) was dissolved in sterile saline. Losartan, dissolved in sterile saline for injections, was a kind gift from Merck. The doses injected in each experimental group are below.

Experimental Protocols

Experiment 1. Changes in IL-1β mRNA expression and IL-1β protein content in the liver were examined in dehydrated rats after an intravenous injection of LPS (2 μg/kg). Each rat received only one injection of LPS, because repeated injections of LPS result in febrile tolerance. The injection of LPS (2 μg/kg iv) was given 30 min after an intravenous injection of either lisinopril (20 mg/kg; lisinopril + LPS group) or saline (saline + LPS group). The control rats received an intravenous injection of saline (vehicle for LPS) 30 min after intravenous saline (vehicle for lisinopril) (saline + saline group). Animals were killed by CO2 stunning followed by decapitation either 2 or 4 h after their second injection (LPS or saline). This procedure was approved by the Committee on the Ethics of Animal Experiments in Tottori University Faculty of Medicine. The liver was quickly removed, frozen, and powdered in liquid nitrogen.

IL-1β mRNA. The hepatic IL-1β mRNA was measured by Northern blot analysis. In brief, total RNA was extracted from each tissue by the guanidinium thiocyanate-phenol-chloroform method (ISOGEN; Nippon Gene). The RNA (20 μg) was separated, according to size, by electrophoresis on 1% agarose gels containing 6.6% formaldehyde, transferred to a nylon membrane, and subjected to hybridization. The probes were labeled with [α-32P]dCTP by the random-priming method (BcaBEST labeling kit; Takara Shuzo) and purified using ProbeQuant G-50 Micro Columns (Amersham Pharmacia Biotech). After hybridization, the membrane was washed under stringent conditions, then subjected to autoradiography. Images of the autoradiographs were taken into a personal computer and analyzed (Windows, Scion Image, Plot Profile (Scion, Frederick, MD)). The density of the IL-1β mRNA fraction was normalized with respect to the β-actin density in each sample and is expressed in arbitrary units.

Probes for Northern blot analysis were prepared by RTPCR. Briefly, cDNA was made from rat spleen total RNA using an oligo(dT)17 primer by means of the RT reaction. Then, PCR fragments derived from rat IL-1β and β-actin mRNA were obtained; the fragments were of 519 and 762 bp, respectively. The primers used for PCR were as follows: IL-1β, sense 5′-CCAGGTATGAGACCCAAGCA-3′, antisense 5′-TCCCGAGATTGTTCT-TCTCC-3′ (24); β-actin, sense 5′-CTATCGGCA-ATGAGCGGTTC-3′, antisense 5′-CTTAGGAGTTGGGTGTGGCT-3′ (24). Each PCR fragment was inserted into a T vector (pT7Blue, Novagen) and cloned in Escherichia coli (XL1-Blue; Stratagene). The T vectors, which contained one of the above two PCR fragments, were selected by sequencing. Then the T vectors were digested with EcoRI and XbaI, and the probes were isolated.

IL-1β content. The liver concentration of IL-1β was measured by ELISA. In brief, after livers had been powdered in liquid nitrogen as mentioned above, each powdered tissue immersed in Iscove’s culture medium containing a cocktail protease inhibitor (Sigma), was mechanically homogenized on ice, using a postmounted laboratory homogenizer (Omni International, Warrenton, VA). Homogenized samples were centrifuged at 10,000 rpm for 10 min at 4°C. Supernatants
were then transferred into a fresh test tube and stored at −85°C until needed for measurement of IL-1β and total protein content. The IL-1β content was measured using a commercial ELISA kit (TFB, Tokyo, Japan) with a lower detection limit of 3 pg/ml. The total protein content was determined using a Bio-Rad protein assay kit. The tissue concentration of IL-1β is expressed as the cytokine content per 100 g protein.

**Experiment 2.** The concentration of IL-1β in the liver was determined in dehydrated rats after injection of LPS (2 μg/kg iv). Losartan (30 mg/kg; losartan + LPS group) or saline (saline + LPS group) was given intravenously to each animal just before the LPS. The control rats received an intravenous injection of saline (vehicle for LPS) immediately after intravenous saline (vehicle for losartan) (saline + saline group). The procedures used for the measurement of the tissue IL-1β concentration were essentially the same as those described for experiment 1.

**Experiment 3.** Blood samples were withdrawn from the cannula previously placed in the jugular vein. The samples were used for the measurement of the plasma concentration of cytokines in dehydrated rats before and after an injection of LPS. Either lisinopril or saline was given intravenously to each animal 30 min before the LPS. Blood samples were taken three times: 1 h before and 2 and 4 h after the injection of LPS. On each occasion, −0.5 ml of blood was withdrawn, collected into a test tube containing 5 μl of Na-heparin solution (1,000 U/ml), and centrifuged at 2,000 rpm for 10 min at 4°C. The plasma was then transferred into a fresh test tube and stored at −85°C until needed for the measurement of cytokines. The plasma concentrations of IL-1β and IL-6 were determined using commercial ELISA kits (TFB) with lower detection limits of 3 and 8 pg/ml, respectively.

**Statistical Analysis**

All results are expressed as means ± SE.

Tissue IL-1 data (experiments 1 and 2; see Figs. 1–3) were analyzed for statistical significance using a one-way ANOVA followed by Fisher’s protected least significant difference test (post hoc test).

For the circulating cytokine data (experiment 3; see Fig. 4), a repeated-measures ANOVA (Macintosh, StatView 4.0) was used to assess the overall effect. In addition, a Student’s t-test with Bonferroni’s correction was used to compare the values obtained in each group at −1 h with those at each subsequent time point.

Details of the results of the various forms of analysis are given in the figure legends. Differences were considered significant at P < 0.05.

**RESULTS**

**Experiment 1: IL-1β mRNA Expression in the Liver**

Figure 1 shows the effect of lisinopril (20 mg/kg iv) on the LPS (2 μg/kg iv)-induced increase in IL-1β mRNA expression in the liver in dehydrated rats (Northern blot analysis). As shown in Fig. 1A, a single intravenous injection of LPS induced a marked increase in IL-1β mRNA expression in the liver at both 2 and 4 h after the injection (saline + LPS group vs. saline + saline group). Treatment with lisinopril exerted an inhibitory effect on this response (lisinopril + LPS group vs. saline + LPS group). In our subsequent semiquantitative analysis of the Northern blot data (Fig. 1B), the inhibitory effect of lisinopril was found to be statistically significant at both 2 and 4 h (P < 0.05).

**Experiment 1: IL-1β Protein Content in the Liver**

Figure 2 shows the effect of lisinopril (20 mg/kg iv) on the LPS (2 μg/kg iv)-induced increase in the liver concentration of IL-1β in dehydrated rats. The saline + LPS group showed a marked increase in the liver concentration of IL-1β compared with the saline + saline group. However, this response was significantly attenuated, at both 2 and 4 h after the LPS injection, by pretreatment with lisinopril (lisinopril + LPS group).

There were no differences in the liver concentration of total protein among the three groups. The above intravenous treatment with lisinopril had no effect on
the control level of hepatic IL-1β in dehydrated rats given an intravenous injection of saline instead of LPS (data not shown).

Experiment 2: IL-1β Protein Content in the Liver

Figure 3 shows the effect of losartan (30 mg/kg iv) on the LPS (2 μg/kg iv)-induced increase in the liver concentration of IL-1β in dehydrated rats. The saline +

LPS group showed an increase in the liver concentration of IL-1β at each time point. When losartan was administered just before the injection of LPS (losartan + LPS group), this LPS-induced effect was significantly attenuated at 2 h after the injection of LPS. Although the mean liver concentration of IL-1β at 4 h after the LPS was lower in the losartan + LPS group than in the saline + LPS group, the effect did not reach significance.

The above intravenous injection of losartan did not have any significant effect on the control level of hepatic IL-1β in dehydrated rats given an intravenous injection of saline instead of LPS (data not shown).

Experiment 3: Plasma Concentrations of IL-1β and IL-6

Circulating IL-1β was not detectable at either 2 or 4 h after the injection of LPS (n = 6). However, as shown in Fig. 4, a single intravenous injection of LPS induced a significant increase in the plasma concentration of IL-6 at each time point. Analysis using a repeated-measures ANOVA indicated that the IL-6 response was significantly attenuated by pretreatment with lisinopril.

An intravenous injection of saline (with or without lisinopril pretreatment) had no effect on the resting plasma level of IL-6 (data not shown).

DISCUSSION

We recently reported that ANG II contributes to the development of LPS-induced fever in dehydrated rats (27). In the present study, we examined whether ANG II is involved in the LPS-induced production of proinflammatory cytokines (i.e., EP). The results showed that a single intravenous injection of LPS induced a
marked increase in the expression of IL-1β mRNA in the liver in dehydrated rats, an effect that was significantly attenuated by pretreatment with an ACE inhibitor. Furthermore, the same ACE inhibitor reduced the LPS-induced increase in the liver concentration of IL-1β protein. These results suggest that ANG II contributes to the LPS-induced production of proinflammatory cytokines such as IL-1β at the transcriptional level. If this is indeed so, the contribution made by ANG II to the production of tissue IL-1β might be responsible, in part, for the development of LPS-induced fever in dehydrated rats, because a direct involvement of IL-1 in the pyrogenic responses to LPS has been demonstrated in the rat (9, 11). These ideas are further strengthened by the present finding that an AT1-receptor antagonist exerted an inhibitory effect on the LPS-induced increase in the liver concentration of IL-1β. The question then arises as to how tissue IL-1β, once its production has been stimulated by ANG II, might be involved in the induction of fever.

In the present study, IL-1β could not be detected in the plasma after an intravenous injection of LPS, as reported previously by Kluger (6). However, the plasma concentration of IL-6 was increased by the injection of LPS, and this response was significantly reduced by treatment with an ACE inhibitor. Several studies have suggested that the LPS-induced production of IL-1β in the tissues results in the local induction of IL-6 and that this enters the general circulation to cause fever (6, 10, 12). Hence, IL-6 is now thought to be a candidate for a circulating pyrogenic cytokine (6). Taken together, the evidence suggests that ANG II may participate in the production of tissue IL-1β that occurs in response to LPS and that this leads to an increase in circulating IL-6, which in turn is partially responsible for the LPS-induced fever. Actually, the IL-1β produced in response to ANG II may also stimulate afferent nerves, leading to the induction of fever. In fact, it has been found that the fever induced by intravenous injection of a low dose (1 μg/kg) of LPS is inhibited by subdiaphragmatic vagotomy (21), suggesting the involvement of vagal afferents in the development of fever. More specifically, Romanovsky (20) noted in a review article that febrile chemical signals such as IL-1 originate in Kupffer cells and bind to appropriate receptors on the hepatic vagus, leading to fever induction. On this basis, part of an LPS-induced fever may be attributable to the action on vagal afferents exerted by hepatic IL-1β after stimulation of its production by ANG II.

It has been suggested that ANG II has proinflammatory properties. Interestingly, in vitro studies have yielded results that lead us to speculate that ANG II is involved in the production of cytokines from LPS-stimulated leukocytes (18, 22). Furthermore, application of ANG II onto cultured mesangial cells results in the production of IL-6 in vitro (15). Collectively, this evidence supports the present finding that ANG II contributes to the LPS-induced production of proinflammatory cytokines in vivo. How then might ANG II contribute to the LPS-stimulated production of proinflammatory cytokines? We know that LPS activates a proinflammatory transcription factor, NF-κB, in monocytes (1, 16). Furthermore, the expression of cytokines is controlled at the transcriptional level through NF-κB (1, 16), and ANG II, too, has been shown to activate NF-κB in monocytes (8). Because the LPS-induced increase in the expression of IL-1β mRNA was attenuated by an ACE inhibitor in the present study, it is possible that activation of NF-κB by LPS is mediated or enhanced by ANG II, leading to an increase in cytokine production. This possibility needs to be examined in the not-too-distant future.

In addition to IL-1 and IL-6, a number of other factors are reportedly involved in the regulation of fever. For example, tumor necrosis factor and interferons are proinflammatory cytokines that may act as pyrogens (3). Conversely, IL-10, vasopressin, α-melanocyte-stimulating hormone, and an arachidonic acid metabolite, epoxyeicosatrienoic acid, act as anti- pyretic substances (7, 17, 25). Furthermore, neither LPS-induced fever (27) nor the IL-1 and IL-6 production in this study was completely abolished by an ACE inhibitor. Hence, it would be unwise to assume that ANG II is the only regulator of IL-1β, and ultimately of fever, or that it is necessarily a direct regulator of this cytokine. We must keep in mind that in addition to ANG II, other mediators and/or regulators are almost certainly involved in the induction of IL-1, IL-6, and fever.

In this study, we used two kinds of LPS: for the lisinopril experiments (experiments 1 and 3), LPS from Difco Laboratories, and for the losartan experiment (experiment 2), LPS from Sigma. We noted that the LPS from Difco produced a significantly greater IL-1β response than that from Sigma at 2 h (but not at 4 h) after the injection of LPS (see Figs. 2 and 3). Therefore, the activities of these LPS would seem to differ to some extent, depending on the source. However, when we compared the febrile responses to these two kinds of LPS, no significant difference was observed; the fever index for a 7-h period (area under the fever curve) was 7.36 ± 0.73°C·h for one LPS (Difco; n = 5) and 7.56 ± 0.93°C·h for the other (Sigma; n = 9) (unpublished observation; Student’s t-test). Thus the difference in biological activity between the two kinds of LPS could be described as “slight,” and we see no reason to think that there would have been any essential difference in the effects of the ACE inhibitor and the AT1-receptor antagonist if we had been able to use the same LPS in all experiments.

The present results represent the first evidence that, in vivo, ANG II and AT1 receptors contribute to the LPS-induced production of proinflammatory cytokines, at least in dehydrated rats. They suggest that dehydration-enhanced fever under infectious conditions may be attributable to an increased production of cytokines, which in turn is due to ANG II (through its action on AT1 receptors). However, although the present results showed a significant attenuation by an ACE inhibitor of the LPS-induced increase in the hepatic IL-1β concentration at both 2 and 4 h after the injection of LPS, the AT1-receptor antagonist exerted a
significant (inhibitory) effect only at 2 h after the LPS injection (see Figs. 2 and 3). Hence, we need to consider the possibility that another ANG II receptor, the type 2 (AT₂) receptor, may be involved in the production of proinflammatory cytokines, too. The effect of an AT₂ receptor antagonist on the LPS-induced production of tissue IL-1β remains to be investigated. Previously, we showed an attenuation of LPS-induced fever by an ACE inhibitor not only in dehydrated rats but also in euhydrated rats, although the effect was greater in the former (27). Thus the action of ANG II in promoting cytokine production is not limited to dehydrated conditions. We think it will be interesting to investigate the significance of the stimulatory action of ANG II on the production of proinflammatory cytokines under euhydrated conditions, using relatively high doses of LPS. Finally, in this study we measured IL-1β mRNA and protein in powdered liver. Therefore, we do not know which cell(s) were the source of the IL-1β. Possible candidates are Kupffer cells, hepatocytes, or blood cells in the liver, including blood-borne phagocytes. However, we think it unlikely that blood cells are the major responsible cells because those cells (the liver should contain erythrocytes as well as leukocytes) produced an undetectable amount of IL-1β in response to LPS (expressed as pg/100 μg protein; unpublished observation). We hope soon to determine which cells in the liver are primarily responsible.

We are grateful to Dr. R. J. Timms for critical reading of the English. We thank Dupont Merck for the kind supply of losartan.

This work was partly supported by the Ministry of Education, Science, and Culture with a Grant-in-Aid for Scientific Research (C12670060).

REFERENCES