Calcitonin gene expression induced by lipopolysaccharide in the rat pituitary

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1Departments of Physiological Chemistry and 2Pharmacology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, 060-0812; and 3Health Sciences University of Hokkaido, Ishikari-Tobetsu, 061-0293 Japan

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Kiriymama, Yoshimitsu, Yasuyuki Nomura, and Yukiko Tokumitsu. Calcitonin gene expression induced by lipopolysaccharide in the rat pituitary. Am J Physiol Endocrinol Metab 282: E1380–E1384, 2002; 10.1152/ajpendo.00453.2001—Procalcitonin (PCT), the precursor protein of calcitonin (CT), has been considered recently as a significant indicator of bacterial infection and sepsis. However, the major source of PCT in sepsis remains unclear. The hypothalamic-pituitary-adrenal axis is activated during sepsis. Moreover, immunoreactive CT (iCT) can be detected in the pituitary. Therefore, we examined the effects of lipopolysaccharide (LPS) administration on CT mRNA expression in the pituitary. After administration of LPS, CT mRNA expression in the pituitary was increased significantly. The increase of CT mRNA was associated with significant elevations of the iCT levels in the serum. These results imply that the pituitary is one of the sources of the serum PCT during sepsis.

procalcitonin; sepsis; lipopolysaccharide

THE BACTERIAL ENDOTOXIN, LIPOPOLYSACCHARIDE (LPS), is thought to be a direct cause of endotoxin shock in gram-negative sepsis. LPS induces a number of shock-state abnormalities that are similar to those observed in sepsis, including fever, hypotension, and multiorgan system failure (5, 14). There are ~500,000 septic episodes each year in the United States, and the mortality rate in patients with septic shock ranges from 35 to 65% (11). Although some proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α have been proposed as indicators of sepsis severity, they are often transiently increased and produced only in local pools (4, 8, 10, 13).

Calcitonin (CT) is a 32-amino acid peptide hormone, which is regulated by serum Ca2+ concentrations and secreted by C cells of the thyroid (26). Its receptor was identified as having C1a and C1b isoforms in rodents (29). Procalcitonin (PCT), the precursor for CT, is a 116-amino acid polypeptide in humans and a 110-amino acid polypeptide in rats, and it consists of amino-procalcitonin, immature CT, and katacalcin. Mature CT is produced by posttranslational processing from PCT and carboxy-terminus amidation. PCT is encoded in CT mRNA (Fig. 1). It has recently been reported that the concentration of serum PCT is increased markedly with sepsis in humans and animals (2, 9, 22, 28, 33). Furthermore, the mortality in septic animals is increased by administration of PCT and decreased by neutralizing antiserum against PCT (24). These results indicate that PCT is a useful predictive marker and plays an important role in sepsis.

LPS activates the hypothalamic-pituitary-adrenal (HPA) axis, and it increases blood concentrations of cytokines, adrenocorticotropic hormone (ACTH), and glucocorticoids (34). In particular, the pituitary plays an important role in the immune regulation through various complex interactions between cytokines and neuroendocrine hormones, which originate at the pituitary and/or peripheral tissues (1, 20). Furthermore, it has been shown that mature CT or CT-like immunoreactivity and the functional receptors for CT exist in the pituitary (17, 27, 30–32). Therefore, we speculated that the pituitary is one of the sources of PCT in sepsis. In the present study, we examined the expression of CT mRNA in the LPS-administered rat pituitary to distinguish the source of PCT in sepsis.

MATERIALS AND METHODS

Animals and treatment. Adult male Wistar rats (250–280 g) were acclimated to standard laboratory conditions (12:12-h light-dark cycle at 22–24°C) with food and water ad libitum. All animal experiments were conducted in accordance with protocols approved by the Animal Care and Use Committee at Hokkaido University. Animals received 5 mg/kg of LPS from Escherichia coli serotype 055:B5 (Sigma Chemical, St. Louis, MO) or saline (same volume) intraperitoneally. The body temperature was monitored periodically by insertion of a thermistor probe into the rectum. Trunk blood stood for 30 min at room temperature and was centrifuged for 5 min at 3,000 rpm to obtain serum. Tissues and serum were stored at −80°C until assay.

ACTH and CT measurement. Concentrations of ACTH and total immunoreactive CT (iCT) in serum were determined by radioimmunoassay (RIA). An ACTH RIA kit was from Nichols Institute Diagnostics (San Juan Capistrano, CA), and a CT RIA kit, recognizing the mature type of CT, was from Mitsubishi Chemicals (Tokyo, Japan).

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RT-PCR and probe preparation. Total RNAs of rat pituitary and hypothalamus homogenates were isolated by acid guanidinium thiocyanate-phenol-chloroform methods (7). Reverse transcriptase reactions were performed on 1 μg of RNA by use of SuperScript II reverse transcriptase (Life Technologies, Rockville, MD) at 42°C for 1 h. One-twentieth of the resulting cDNAs was subjected to PCR reaction with the Expand High Fidelity PCR system (Roche Molecular Biochemicals, Mannheim, Germany), with 200 nM of each dNTP and 300 nM of each forward and reverse primers for CT, CTR, IL-1β, TNF-α, IL-6, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers were as follows:

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<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>CT</td>
<td>forward TGGAGCAGGAGGAGAAGAGG</td>
<td>reverse GAGGACCTAGTGGCACAAT</td>
</tr>
<tr>
<td>CTR</td>
<td>forward GTTGAAGGTGTTGGCCCAATGGA</td>
<td>reverse CCGTGGAAATGAATCAGAGG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>forward CCTTCTTTTTCTTCATCTTGTG</td>
<td>reverse ACCGTCTTCTTCTTCCTCT</td>
</tr>
<tr>
<td>IL-6</td>
<td>forward CTGGAGCTAGTGTGTTGACG</td>
<td>reverse TCTGAAATGCTCTGCTTGTG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>forward GAAAGCATGATCCGAGATG</td>
<td>reverse AAGAGACCTGCCCCGGGAC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>forward AACCCCATCACCATCTCCAG</td>
<td>reverse AGGGCCATCCACAGTCTTCT</td>
</tr>
</tbody>
</table>

GAPDH was used as a control for the quality of the cDNA for each PCR reaction. The cycle consisted of denaturation (45 s at 94°C), annealing (45 s at 59°C), extension (1 min at 72°C), and 7 min of final extension at 72°C after amplification. The cycles were 30 for CT and cytokines, 35 for CTR, and 22 for GAPDH. PCR products were cloned using the pGEM-T vector system I (Promega, Madison, WI). The identity of the PCR products was verified by sequencing. Probes for Northern blotting were labeled using cloned PCR products as templates. Briefly, PCR was performed using 200 nM dATP, dGTP, and dTTP, and 0.1 mCi of [32P]dCTP instead of 200 nM of each dNTP.

Northern blot analysis. Northern blot analysis was performed by using a standard protocol. Briefly, 20 μg of total RNA were fractionated on 1% denaturing formaldehyde-agarose gels and then transferred to an Optitran-reinforced nitrocellulose filter (Schleicher & Schuell, Keene, NH). After baking at 80°C, the membranes were prehybridized for 2 h at 42°C in 50% formamide, 5× saline-sodium phosphate-EDTA (SSPE) buffer, 5× Denhardt’s solution, 0.5% SDS, and 50 μg/ml salmon sperm DNA and then hybridized for 16 h at 42°C with [32P]-labeled cDNA probes. After standard washing steps, the membranes were subjected to autoradiography. The blot was then reprobed with a GAPDH probe to confirm mRNA integrity.

Statistical analysis. Data are presented as means ± SE. Statistical significance was performed using one-way ANOVA followed by Dunnett’s test unless otherwise indicated.

RESULTS

We analyzed the expression of CT mRNA or CTR mRNA in the unstimulated rat pituitary and hypothalamus by RT-PCR. The PCR product size was 228 base pairs (bp) for CT, 545 bp for C1a, 656 bp for C1b, and 361 bp for GAPDH. CT mRNA was detected in both the pituitary and the hypothalamus. CT mRNA in the pituitary was much more abundant than that present in the hypothalamus. C1a subtype mRNA was expressed in both the pituitary and the hypothalamus, whereas C1b subtype mRNA was expressed only in the hypothalamus (Fig. 2).

As shown in Fig. 3A, administration of 5 mg/kg LPS caused the body temperature to decrease, starting 1 h after administration and reaching a maximum at 1.5 h (37.00 ± 0.06°C). Within 2 h after LPS administration, the body temperature began to increase gradually, reaching a maximum at 7.5 h (38.57 ± 0.05°C) that lasted for 12 h (38.48 ± 0.1°C). The body temperature was not affected significantly by administration of saline. Moreover, serum levels of ACTH were measured by RIA (Fig. 3B). LPS administration resulted in a significant increase in levels of ACTH in serum, reaching 203.3 ± 42.6 pg/ml at 1.5 h. Levels then decreased after 3 h and dropped to 35.7 ± 10.9 pg/ml at 12 h. ACTH in serum was not detected in the saline-administered rats. We also examined the expression of proin-
flammatory cytokine mRNAs in the pituitary of LPS-administered rats. The expression of IL-1β, IL-6, and TNF-α mRNAs increased to a maximum at 3 h. IL-1β mRNA expression was sustained for 9 h and then decreased at 12 h after LPS administration. On the other hand, the decrease in TNF-α mRNA expression began at 9 h. Compared with the expression of IL-1β and TNF-α mRNAs, the expression of IL-6 mRNA returned to basal levels at 9 h. The mRNA of cytokines in slight amounts can be detected in saline-administered rats at all time points (Fig. 3).

An increase in serum levels of total iCT was observed at 6 h after LPS administration (51.3 ± 3.5 pg/ml) and continued until 12 h (66.7 ± 9.2 pg/ml). Serum levels of total iCT in the saline-administered rats ranged from 40 to 45 pg/ml at all time points (Fig. 4). To investigate the effect of LPS administration on CT mRNA expression in the pituitary, total RNA in the pituitary was subjected to Northern blot analysis. Similar to serum levels of iCT, the expression of CT mRNA was increased beginning at 6 h and sustained until 12 h after LPS administration. A small amount of CT mRNA was expressed in the saline-administered rats (Fig. 5). CT mRNA in the hypothalamus was not detected by Northern blotting analysis (data not shown).

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**Fig. 3.** Physiological effects of lipopolysaccharide (LPS) on rats. A: time courses of rat body temperature after LPS (●) or saline (○) administration. Data represent means ± SE of 12–20 animals in each group. *P < 0.05 and **P < 0.01 compared with saline at the same time point. B: time courses of serum ACTH levels after LPS (●) or saline (○) administration. Data are means ± SE of 3 animals in each group. Statistical analysis was performed by 1-way ANOVA followed by a Fisher's protected least significant difference test. *P < 0.01 compared with saline at the same time point. C: RT-PCR analysis of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α mRNA expression in the pituitary after LPS or saline administration.

**Fig. 4.** Time courses of serum CT levels after LPS (●) or saline (○) administration. Data are means ± SE of 4–6 animals in each group. *P < 0.05 compared with saline at the same time point.
DISCUSSION

Many inflammatory mediators and acute-phase reactants, such as proinflammatory cytokines or C-reactive protein, have been used as indicators of sepsis, but these substances are not specific for sepsis: they are increased in inflammation without infection, such as trauma (4, 8, 10, 13). However, PCT is selectively induced in sepsis or multiorgan dysfunction syndrome (2, 9, 22, 28, 33). Therefore, PCT is now considered to be the specific indicator of sepsis.

PCT is physiologically produced as the precursor molecule of CT in the parafollicular cells of the thyroid gland. However, PCT in serum is detected in thyroidectomized patients during bacterial infection (2). Hence, another organ is considered as the source of PCT in sepsis. As it has been reported (17, 27, 30–32), mature CT peptide or CT-like immunoreactivity and functional receptors for CT are present in the pituitary and hypothalamus, and CT and its receptor mRNAs were expressed in the normal pituitary and hypothalamus (Fig. 2). Although a specific receptor for PCT has not been identified, an immunoreactive protein whose molecular weight is close to PCT binds to CTR (3, 16). These findings suggest the existence of an ultra-short regulatory loop between the hypothalamus and the pituitary and an autocrine regulation within both tissues by PCT and/or CT. CT mRNA was much more abundant in the pituitary than in the hypothalamus. Furthermore, the pituitary directory controls physiological conditions by secreting peptide hormones into the blood. Therefore, we considered that the pituitary might be one of the sources of serum PCT in sepsis.

Because the body temperature, ACTH, and proinflammatory cytokines are increased in sepsis (18, 19, 24), we confirmed that LPS (5 mg/kg ip) induced an increase in body temperature, serum ACTH levels, and mRNA expression of IL-1β, IL-6, and TNF-α in the pituitary (Fig. 3, A–C). Thus this dose of LPS was used for the model of sepsis in the present study. After LPS administration, serum levels of total iCT were significantly elevated (Fig. 4). Although serum PCT levels were limited from normal to minimally elevated in humans and rodents (23, 25). Therefore, increased serum levels of iCT in LPS-administered rats are speculated to be PCT. Moreover, the expression of CT mRNA in the pituitary was markedly increased from 6 h and lasted to 12 h after LPS administration (Fig. 5). The time course of increased CT mRNA expression by LPS was parallel to that of increased serum iCT by LPS. These results imply that the pituitary is one of the sources of increased serum PCT in sepsis. Recently, increased CT mRNA in sepsis models was detected in other tissues and the peripheral blood cells by RT-PCR (23, 35). CT mRNA in the mononuclear cells was stimulated by LPS as well as proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α (25). In addition, PCT decreased LPS-induced TNF-α (15, 21). Because LPS stimulated CT mRNA in addition to IL-1β, IL-6, and TNF-α mRNAs in the pituitary, an interaction between PCT and/or CT and cytokines may exist in the pituitary. In particular, macrophage migration inhibitory factor (MIF), one of the first cytokines to be discovered, has been shown to have a similarity to PCT during sepsis. 1) MIF is secreted by inflammatory stimuli, cytokines, and stress-induced activation of the HPA axis and is considered to be one of the septic markers (12). 2) MIF is induced in the pituitary and monocytes/macrophages during sepsis (12, 24). 3) Neutralization of MIF protects from septic shock (6). Therefore, it is tempting to speculate that the correlations between PCT and MIF exist in the pituitary and/or peripherals during sepsis.

In summary, we have demonstrated that CT mRNA was expressed in the rat pituitary. Moreover, we have shown that LPS stimulated the pituitary, followed by an increase in serum iCT and CT mRNA induction in the pituitary.

REFERENCES