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Short Communication

LPS induces stefin A3 expression in mouse primary cultured glial cells

Toru Hosoi[a,b,1], Sawako Suzuki[a], Yasunobu Okuma[a,c,*,2], Akiko Kawakami[e,3], Naoki Ogawa[e,3], Koichiro Ozawa[b,1], Yasuyuki Nomura[a,d,4]

[a]Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan
[b]Department of Pharmacotherapy, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima 734-8551, Japan
[c]Department of Pharmacology, Faculty of Pharmaceutical Sciences, Chiba Institute of Science, 15-8 Shiomi-cho, Choshi, Chiba 288-0025, Japan
[d]Daiichi University, College of Pharmaceutical Sciences, Fukuoka 815-8511, Japan
[e]Department of Research and Development, GeneticLab Co., Ltd., Sapporo 001-0027, Japan

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Abstract

We searched for a gene that is up-regulated in response to LPS at a later time point in primary cultured glial cells. Using a Gene Chip Probe Array, we identified stefin A3, which is known as a cysteine protease inhibitor. As assessed by RT-PCR, we observed a time-dependent (2 to 48 h) up-regulation of stefin A3. The results indicate that stefin A3 is involved in infection and inflammation at a later time point.

Theme: Disorders of the nervous system
Topic: Infectious diseases

Keywords: Inflammation; Cysteine protease inhibitor; Gene Chip Probe Array

IL-1β, IL-6, and TNF-α are pro-inflammatory cytokines produced not only in the immune system (e.g., by lymphocytes and macrophages) but also in the brain (by neuronal and glial cells). Increased expression of pro-inflammatory cytokines in the CNS has been observed in a number of different inflammatory and degenerative conditions [6,9,12,16,17,20]. Blood–brain barrier permeability has been reported to increase in a state of sepsis [5], and bacteria have been observed to enter the CNS following direct interaction with the luminal side of the cerebral endothelia, which constitute the blood–brain barrier [15]. Thus, bacteria directly act on the CNS during infection and induce inflammation. Cytokines are mainly produced by astrocytes and microglia in inflammatory conditions of the CNS [4,7,11,13,14]. To investigate the role of glial cells in inflammation, we searched for a gene that is induced by lipopolysaccharide (LPS), the cell wall component of Gram-negative bacteria. LPS has been reported to bind toll-like receptor 4 and activate the innate immune system [2]. Thus, in the present study, we used LPS as a model system to investigate the immune function of glial cells. Most pro-inflammatory cytokines are induced within 12 h, and there have been few studies in which a gene that is induced at a later time point was investigated. Therefore, in the present study, we focused on a gene that is specifically induced at a later time point. We searched for a gene that is induced by short (4 h) and long (48 h) exposure to LPS using Gene Chip Probe Arrays. We found that mouse stefin A3 gene [18] was gradually up-regulated from 4 to 48 h in response to LPS in mouse primary cultured glial cells.

Primary cultured glial cells were prepared from whole brains of neonatal (<24 h) C57BL/6 mice as described previously [10]. The cells were allowed to grow to con-

* Corresponding author. Department of Pharmacology, Faculty of Pharmaceutical Sciences, Chiba Institute of Science, 15-8 Shiomi-cho, Choshi, Chiba 288-0025, Japan. Fax: +81 479 30 4673.
E-mail address: okumay@cis.ac.jp (Y. Okuma).
[1] Fax: +81 82 257 5332.
[2] Fax: +81 479 30 4673.
[3] Fax: +81 11 756 8747.
[4] Fax: +81 92 553 5698.

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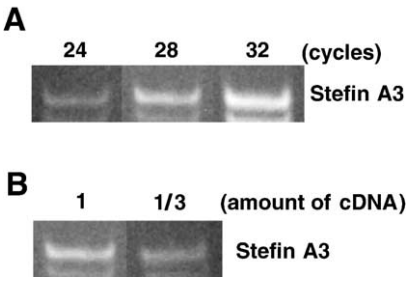

Fig. 1. Standardization data of RT-PCR for stefin A3. (A) RT-PCR for stefin A3 in the indicated cycles. LPS (48 h)-treated samples are used. (B) Comparison of input cDNA (1 and 1/3) and resulting PCR product at 28 cycles. LPS (48 h)-treated samples are used.

fluency (10 days) in DMEM medium with 10% FCS, 100 units/ml penicillin G, and 100 μg/ml streptomycin (GIBCO BRL). All of the cultured cells were kept at 37 °C in 5% CO_2/95% air. Subsequently, mixed glial cells were shaken at 120 rpm for 18 h and cultured again for 1 week in 60-mm dishes and were then used in the following experiments. At this point, the astrocyte cultures were routinely >95% positive for glial fibrillary acidic protein, and ~3% of the cells were microglia, based on positive ED1 (anti-macrophage/microglia monoclonal antibody) staining. For the Gene Chip Probe Array, primary cultured glial cells were treated with LPS for 4 and 48 h, and total RNA was isolated using TRI REAGENT™ (SIGMA). Two independent sets of the glial cells were pooled (25 μg total RNA/each sample) and analyzed using GeneChip Mouse Expression Set 430 A (Affymetrix). We entrusted the analysis to GeneticLab Co., Ltd. (Japan). For RT-PCR analysis, total RNA was isolated using TRI REAGENT™ (SIGMA), and cDNA was synthesized as described previously [8]. For PCR amplification, 1.2 μl of cDNA was added to 12 μl of a reaction mixture containing 0.2 μM of each primer, 0.2 mM of dNTP mix, 0.6 U of Taq polymerase, and 1× reaction buffer. PCR was performed in a DNA Thermal Cycler (Perkin-Elmer 2400-R). The primers used were as follows: Stefin A3 upstream, 5′-GAG CAA ACC AAT GAG AAA TA-3′; stefin A3 downstream, 5′-CAA GGA GAA GAA TCA GGT CA-3′; GAPDH upstream, 5′-AAA CCC ATC ACC ATC TTC CAG-3′; and GAPDH downstream, 5′-AGG GGC CAT CCA CAG TCT TCT-3′. The PCR products (10 μl) were resolved by electrophoresis, and the gel was stained with ethidium bromide. In the initial experiments, the amount of each amplified product was integrated and plotted graphically against the number of PCR cycles to determine whether the increase in intensity of the amplified product was linear with respect to the number of PCR cycles. As shown in Fig. 1A, we observed a linear relation of stefin A3 mRNA expression between 24 to 32 cycles. Moreover, at 28 cycles, PCR produced a linear relation between the amount of input cDNA and the resulting PCR product (Fig. 1B). Thus, we chose 28 cycles to investigate stefin A3 expression in the present study. To compare the expressions of mRNAs in different experimental group, the amount of

Table 1
Gene Chip Probe Array of primary cultured glial cells treated with LPS

	4 h	48 h
IL-6	181.0	39.4
IL-1β	59.7	32.0
TNF	97.0	3.2

Primary cultured glial cells were treated with sterilized water (as control cells) or with LPS for the indicated times and then analyzed using Gene Chip Probe Array. Quantitative changes in gene expression are expressed as signal log ratio in statistical algorithms. Data are expressed as fold change compared with control cells. Fold change = $2^{\text{signal log ratio}}$ (signal log ratio ≥ 0) or fold change = $(-1) * 2^{-(\text{signal log ratio})}$ (signal log ratio < 0). LPS induced pro-inflammatory cytokines in primary cultured glial cells.

Table 2
Gene Chip Probe Array of primary cultured glial cells treated with LPS

	4 h	48 h
Stefin A3	1.1	90.5
Cystatin B	1.4	1.4
Cystatin C	1.1	0.7
Cystatin F	1.1	0.2
Kininogen 1	0.5	3.0

Primary cultured glial cells were treated with sterilized water (as control cells) or with LPS for the indicated times and then analyzed using Gene Chip Probe Array. Quantitative changes in gene expression are expressed as signal log ratio in statistical algorithms. Data are expressed as fold change compared with control cells. Fold change = $2^{\text{signal log ratio}}$ (signal log ratio ≥ 0) or fold change = $(-1) * 2^{-(\text{signal log ratio})}$ (signal log ratio < 0). LPS markedly induced stefin A3 mRNA expression in primary cultured glial cells.

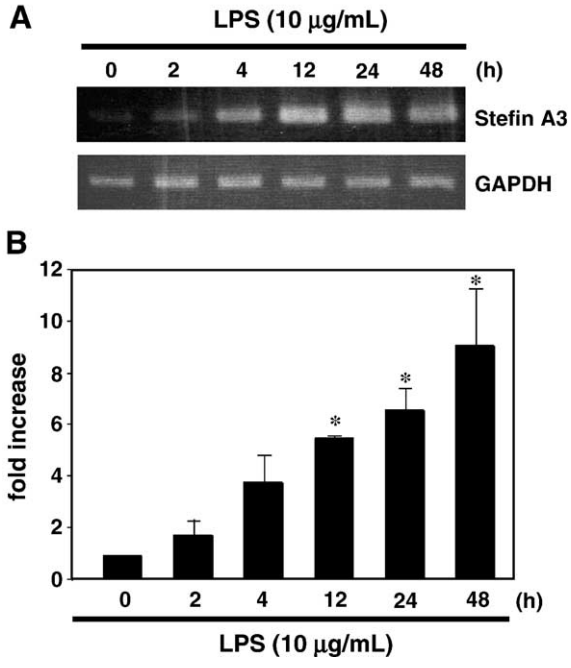

Fig. 2. LPS induced stefin A3 expression in mouse primary cultured glial cells. (A) Glial cells were stimulated with LPS (10 μg/ml) for indicated times, and RT-PCR was performed. (B) The amounts of stefin A3 mRNA are expressed as ratios of densitometric measurements of the samples to the corresponding GAPDH internal standard. Values are presented as means ± SE (n = 3 per group).