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Inhibitory effects of 17β-estradiol on inflammatory cytokines released from lipopolysaccharide stimulated microglia

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Abstract Acute brain insults are described to be associated with activation of microglia, which produces cytokines. Inappropriate activation of microglia contributes to progressive neuronal damage and neurodegeneration. 17β-estradiol (17β-EST) is reported to be effective to prevent or to treat neurodegenerative diseases. Microglia have estrogen receptors, so that 17β-EST might affect inflammatory cytokines. In the present study, the concentration of cytokines in the supernatant of microglial culture activated by lipopolysaccharide with or without 17β-EST was measured. Interleukin-1β, tumor necrosis factor α and interleukin-6 were increased in the supernatant steeply from 3 hrs after lipopolysaccharide stimulation. Estrogen could restrain the release of those cytokines to approximately half of each level at least for 24 hrs, respectively.

In conclusion, 17β-EST inhibited the release of inflammatory cytokines from lipopolysaccharide stimulated microglia. 17β-EST might play important roles in preventing acute brain damages via suppression of inflammatory cytokines.

INTRODUCTION

Microglia are immuno-reactive cells in the central nervous system (CNS), which is activated by acute brain insults and chronic brain diseases .[1-3] When microglia are activated inappropriately by brain insults such as infarction, neuronal cells are injured .[4,5] Because the activated microglia release massive dose of nitric oxide[6,7] and inflammatory cytokines, such as tumor necrosis factor α (TNFα), interleukin-1β (IL-1β) and interleukin-6 (IL-6)).[8,9] On the other hand, the activation of microglia is regulated or suppressed by immuno-suppressants, such as corticosteroid .[10]

Estrogen affects microglial phagocytic and acute inflammatory functions.[11] Recently, it has been found that microglia have estrogen receptors.[12] In the present study, we studied the effect of 17β-EST, a human intrinsic estrogen, on the release of inflammatory cytokines induced by lipopolysaccharide (LPS) from primary cultured microglia.

MATERIALS AND METHODS

Preparation of primary cell culture

Isolation of microglia was carried out according to the method described by Si et al.[13] Microglia were isolated from primary cell cultures of newborn Wistar rat brains. Whole brains gathered from neonatal rats (1-2 day old) were mechanically triturated

and homogenized using Pasture pipette in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, NY, USA) with 10% fetal calf serum (FCS; Filtron, Brooklyn, Australia), and the mixed cell suspension was plated in a flask (three brains per SoLo flask of 185 cm^2; Nunc Naperville, IL, USA). Through the above procedures mother cultures were prepared. Then these mother cultures were incubated at 37 ℃, 95% humidity and 5% CO_2 for 2 weeks with replacing the medium every week. Microglia growing on the top of the confluent cell monolayer were removed by shaking gently under microscopic observation. Floating cells in the supernatant were collected, centrifuged and plated into 96-well culture-plates (Iwaki, Tokyo, Japan). One hour later, the medium was replaced and the cells were washed twice with DMEM containing 10% FCS to remove non-adherent cells. The remaining microglia were allowed to stabilize for 1 day in DMEM containing 10% FCS before the cells were used for experiments. The purity of microglial cultures (90-96%) was confirmed using lectin GSA-I-B4 (Sigma, St. Louis, MO, USA) stain.[14)]

Measurement of microglial proliferation

LPS-stimulated microglia with or without 17β-EST were counted at 24 hrs by the two methods as follows.

The first method; microglia (2×10^4 cells/well) were stimulated by 1 μg/ml LPS with or without 10 nM of 17β-EST and were incubated in DMEM containing 10% FCS for 24 hrs. Thereafter, they were trypsnized at 37 ℃ for 1 hour. Then trypan blue was added into the supernatants containing microglia. The number of cells was counted through a microscope using a counting chamber.

The second method; the number of cells was also estimated by a cell proliferation assay system (Premix WST-1 Cell Proliferation Assay System; Takara, Tokyo, Japan), using an absorbance indicator (450 nm wave length) with an established standard absorption curve 24 hrs after LPS stimulation.

Cell preparation for electron microscopy

Cells were embedded in Epon 812. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate and analyzed with a 200CX transmission electron microscope (JEOL, Tokyo) operating at 80kV.

Experimental protocol

The cell culture medium and the environment were same as before. Microglia

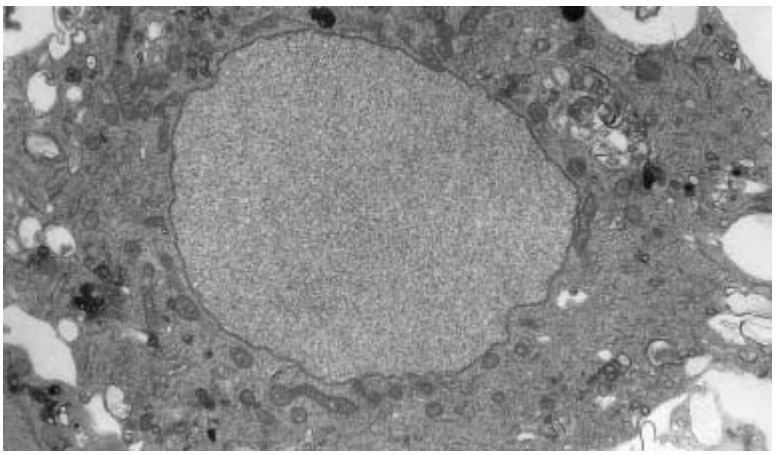


Fig. 1 Electron microscopic appearance of microglia
Microglia were gathered soon after they were isolated from primary culture. Electron microscopy reveals that there is a little chromatin in the nucleus and mitochondria and lysosomes can be seen in the cytoplasm. ×7500

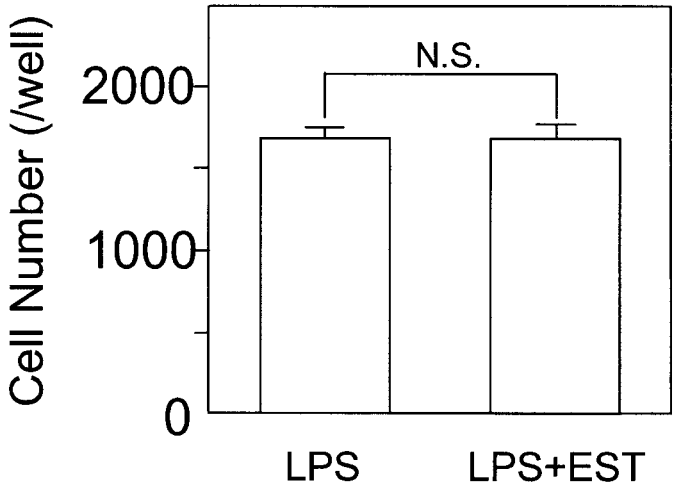


Fig. 2 Cell count of microglia
Microglia were incubated and measured at 24 hrs after lipopolysaccharide (LPS) stimulation. 17β-estradiol (17β-EST) did not influence LPS stimulated microglial number. Values represented mean ± S.E. of 14 experiments.

were stimulated by 1 μg/ml LPS (LPS derived from *Salmonella enteritidis*; Sigma, St. Louis, MO, USA). Medium/dimethylsulfoxide (DMSO; solvent of 17β-EST) or 10 nM 17β-EST (Sigma, St. Louis, MO, USA) solved in the same solvent was added into the each medium 2 hrs after LPS stimulation. The concentration (10 nM) of 17β-EST was set within the range of physiological surge because 17β-EST has an optimum concentration.[11] The supernatant of the cultured microglia (2×10^4 cells/well) was collected from each well at the time points of 0, 0.5, 3, 5, 8, 10, 12 and 24 hrs after the LPS stimulation.

Cytokine assay

The concentrations of TNFα, IL-1β and IL-6 in the supernatant of the microglia culture were determined by enzyme-linked immunosorbent assay (ELISA) kits (TFB, Tokyo, Japan) with a microplate reader (TFB, Tokyo, Japan). The detection limits of TNFα, IL-1β and IL-6 using these kits were approximately 4 pg/ml, 10 pg/ml and 8 pg/ml ,respectively. Each value was standardized by the cultured microglia numbers (2×10^4 cells/well).

Data analysis

The data were expressed as mean ±S.E. of 7 wells. For statistical analysis of the data, unpaired Student t-test and two-way ANOVA followed by Scheffe's multiple comparison procedure were used. P values less than 0.05 were considered to be statistically significant.

RESULTS

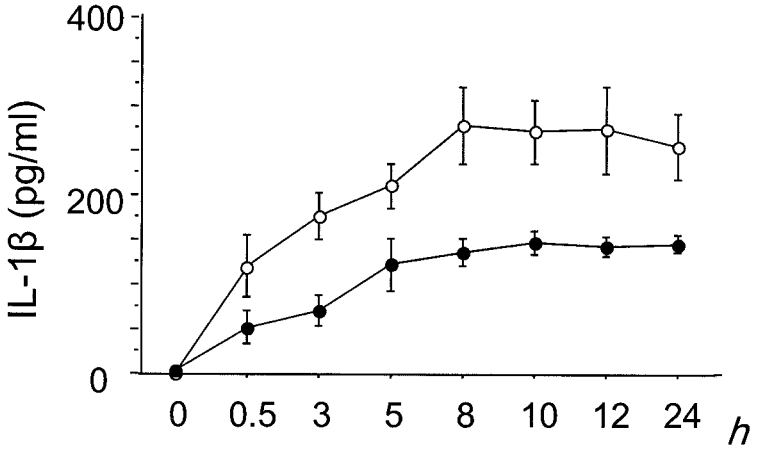
General appearance and cell count of microglia

Microscopic findings showed that the shape of microglia stimulated by LPS with or without 17β-EST for 7 hrs was changed from ramified (resting) to ameboid (reactive) form. 17β-EST did not change the morphologic shapes of microglia from those stimulated by LPS. Thereafter, microglia were ramified and came into resting states, finally. Microglia were also identified by an electron microscopy. Microglia have many lysosomes and phagosomes in the cytoplasm, and some of them revealed dendritic shapes. Microglia appeared different from fibroblasts and nerve cells (Fig. 1).

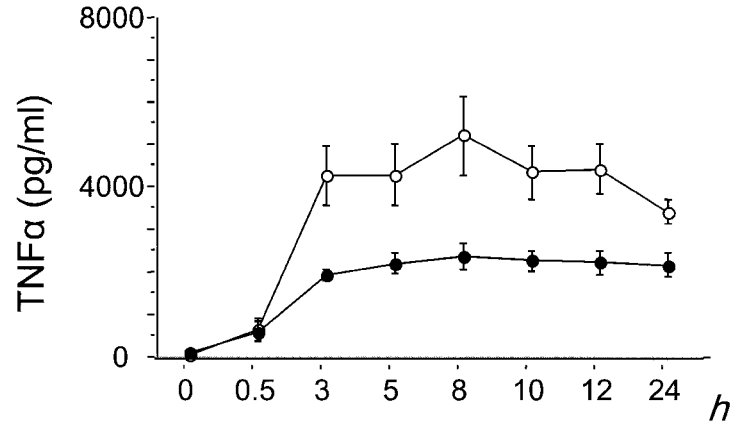
Cell numbers of microglia were not statistically different between the wells with and without 17β-EST, 24 hrs after LPS stimulation. The numbers of microglia es-

timated by the assay system 24 hrs after LPS stimulation were not statistically different between the two groups (Fig. 2). The number of microglia was 1715±32 (mean±S.E., n=14) in LPS group and 1711±73 in LPS+EST group.

Table 1 shows the changes of IL-1β, TNFα and IL-6 for 24 hrs after stimulation by LPS with or without 17β-EST. Figure 3A, B and C show the sequential changes of IL-1β, TNFα and IL-6 after stimulation by LPS with or without 17



3－A



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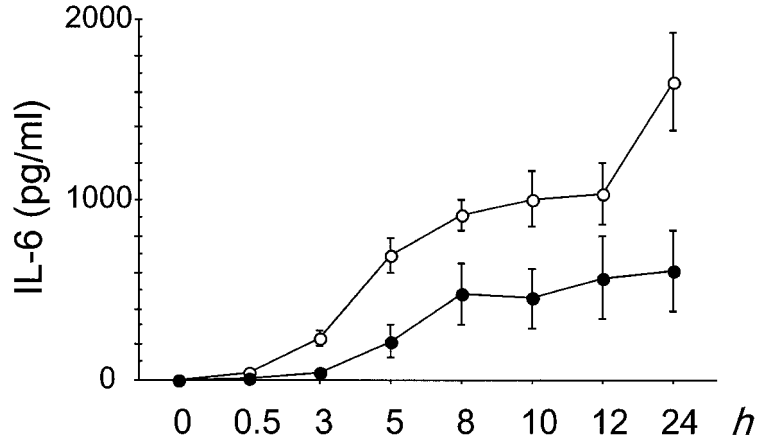

3－C

Fig. 3 Effects of 17β-EST on LPS-induced microglial activation
Response curves of cytokines released from microglia (n=7). Microglia were incubated with 1 μg/ml LPS at 0 hour, and 17β-EST was added at 2 hrs (closed circles; with 17β-EST, opened circles; without 17β-EST). A: IL-1β (interleukin-1β); B: TNFα (tumor necrosis factor α); C: IL-6 (interleukin-6) Data are expressed as mean ± S.E. of 7 wells.

Table 1. Effects of 17β-EST on microglial cytokine production
Microglia were stimulated at 0 hour by lipopolysaccharide (LPS) and 17β-estradiol (17β-EST) was added at 2 hrs. Data are expressed as mean ± S.E. of 7 wells. *p<0.05, **p<0.001 vs without 17β-EST.

hours	0	0.5	3	5	8	10	12	24
IL-1β (pg/ml)								
LPS alone	0	110±35	175±26**	210±25*	278±44**	271±36**	273±49*	254±36*
LPS+EST	0	49±19	67±17	119±30	132±15	143±13	139±11	141±10
TNF-α (pg/ml)								
LPS alone	64±31	647±265	4263±686**	4278±729**	5202±928*	4343±626*	4403±591**	3402±288*
LPS+EST	64±31	522±239	1883±135	2145±229	2294±302	2203±242	2165±286	2105±272
IL-6(pg/ml)								
LPS alone	1±1	42±16	234±40*	697±94**	912±85*	1005±150*	1031±171**	1650±272*
LPS+EST	1±1	5±2	42±10	218±90	481±174	456±166	566±229	606±225

*P<0.55, **P<0.001

β-EST, respectively.

LPS activation of microglia

IL-1β was steeply increased from baseline at 30 min, which was lasted for at least 24 hrs after the application of LPS (Fig. 3A). TNFα was increased at 3 hrs and the level lasted for at least 24 hrs (Fig. 3B). IL-6 was continuously increased from 3 hrs to 24 hrs after LPS stimulation (Fig. 3C). The maximum concentration of IL-6 was recorded at 24 hrs, while those of IL-1β and TNFα were tended to decline at 24 hrs after LPS stimulation (Fig. 3 A, B and C). The concentration of proinflammatory cytokines (IL-1β and TNFα) reached to the maximum levels at 8 hrs. On the other hand, IL-6 increased continuously from early to late phases (24 hrs).

17β-EST attenuated release of cytokines from LPS-stimulated microglia

17β-EST restrained the amount of cytokines released from LPS-stimulated microglia. Estrogen significantly restrained the quantity of IL-1β and TNFα and IL-6 from 3 hrs to 24 hrs (Fig. 3A, B and C). Thus 17β-EST effectively inhibited cytokine release in the acute phase reaction after LPS-stimulation.

DISCUSSION

Microglia are found throughout CNS, comprising between 5% and 15% of all brain cells.[15] It has been reported that microglia activation is marked by proliferation and migration to the site of injury.[16,17] When microglia are activated, they produce inflammatory cytokines and play an important role not only in chronic brain disease including Alzheimer's disease and amyotrophic lateral sclerosis[18,19], but also in acute brain injuries including infarction, hemorrhage, hypoxia.[16,17,20] Microglia are involved in both neuroprotective and neurodestructive function according to brain states, because microglia have numerous functions, such as phagocytic activity, immune regulatory functions, and the action on formation of cytokine network by releasing acute response cytokines (TNFα, IL-1β and IL-6) and NOx.[21–23] These substances affect neurons in CNS. The control of microglia activation rouses clinical interests because they affect on maintenance of microenvironment and trigger cytokine network in CNS.[10,24,25] However, pathophysiology induced by microglial activation and the mechanism of protection against injuries during early phase have not been fully understood.

It has been reported that if neurodestructive function is exceeded at early phase in brain insults, neuronal cells are massive-

ly injured.[2,21] Therefore, it will be necessary to find some substances to control or suppress the activated microglial functions beside corticosonteroid[10], which may have uncertain effects on using in brain insulted patients.[26,27] The inhibitor of microglia is considered to be useful to control the brain conditions and may become the potential therapeutic drug for brain insults. Recently, it is reported that microglia have estrogen receptors[12] and 17β-EST might affect to prevent neurodestruction. Therefore, 17β-EST would be a candidate to protect the brain against acute brain insults.

The present study demonstrates that 17β-EST has anti-inflammatory effects on microglial activation in vitro, which is shown by suppression of proinflammatory (IL-1β and TNFα) and inflammatory (IL-6) cytokines production (Table 1, Fig. 3 A, B and C). Proinflammatory cytokines, IL-1β and TNFα appear to exacerbate cerebral ischemia damage.[28,29] On the contrary, over expression of IL-1 receptor antagonist reduces brain damage[30,31], and administration of soluble TNF-receptor inhibition or anti-TNFα monoclonal antibody also reduce brain damage.[32,33] Therefore, present results describe a novel mechanism by which a human estrogen, 17β-EST could prevent neuronal damage in the brain insults, such as ischemic stroke.

Inflammatory cytokine IL-6 also appears to exacerbate cerebral ischemic damage. It is supported by the fact that elevation of initial CSF IL-6 concentrations significantly correlated with magnetic resonance imaging infarct volume at 2 to 3 months in human.[34] But it is also reported that IL-6 is an important endogenous inhibitor in neuronal death, because intracerebroventricular injection of recombinant IL-6 significantly reduces ischemic brain damage in rats.[35] The discrepancy of these reports may be explained by the facts that the lager the brain infract volume is, the higher the concentrations of CSF IL-1β and TNFα are, and high concentration of IL-6 is induced by these proinflammatory cytokines. Probably, the present fact that the suppression of IL-6 by 17β-EST to the approximately half of the control may be preferable when the both situations are exist.

Estrogen restrained both proinflammatory cytokines and inflammatory cytokine production in the present study (Table 1, Fig. 3A, B and C). However, it could not be elucidated that this inhibitory effects of estrogen was generated in the nucleus where generally estrogen acted on, or in the cytoplasm.[11] It has been reported that the pathway of releasing cytokines activated by LPS differs from that activated by IL-1β.[36] Therefore, it will be necessary to identify the signal transduction pathway triggered by LPS in microglia.

The concentration (10 nM) of 17β-EST was set within the range of human physiological surge, because estrogen has optimum concentrations (0.1-10 nM) to attenuate microglial superoxide release, phagocytic activity and inducible nitric oxide synthase expression.[11] Although the different concentration of 17β-EST should be examined, I have studied by the concentration in which the maximum effects on the activated microglia were obtained.

To concern the clinical application of 17β-EST, it was added to the medium of microglia at 2 hrs after the LPS stimulation. The present results mean that 17β-EST is applicable at least 2 hrs after the brain insults in the clinical practice.

Currently, some investigators report that estrogen makes neuronal cell apoptosis with adhering FasL gene in the nucleus.[37,38] In our study, 17β-EST did not affect the number of LPS-stimulated microglia (Fig. 2.), so that 17β-EST itself does not cause microglial apoptosis.

In conclusion, our findings indicate that 17β-EST inhibited inflammatory cytokines released from LPS-stimulated microglia. 17β-EST might play important roles in preventing acute brain damages via suppression of inflammatory cytokines.

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