

**No Involvement of Acid Sphingomyelinase in the Secretion of IL-6 from
Alveolar Macrophages in Rat**

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Abstract

Chronic lung disease (CLD) of the newborn is a major problem in neonatology. Activation of alveolar macrophages has been implicated in the pathogenesis of CLD. Acid sphingomyelinase (ASM) responds to diverse cellular stressors, including lipopolysaccharide (LPS) stimulation. Recently, functional inhibitors of acid sphingomyelinase (FIASMA) have been described as a large group of compounds that inhibit ASM. Here, we used maternal intra-peritoneal LPS injection to model CLD in the infant rat lung. Using this model, we studied ASM activity in the infant rat lung and the effects of FIASMA on release of interleukin-6 (IL-6) from LPS-stimulated alveolar macrophages. Maternal exposure to LPS non-significantly increased ASM activities in the infant rat lung. FIASMA significantly decreased ASM activity of LPS-stimulated alveolar macrophages. In addition, some FIASMA suppressed the release of IL-6 from LPS-stimulated alveolar macrophages during the early response phase. However, FIASMA did not suppress the release of IL-6 from LPS-stimulated alveolar macrophages. From our study, we could not confirm that ASM activation is responsible for LPS-related pathogenesis of CLD and release of IL-6 from LPS-stimulated alveolar macrophages.

Introduction

Chronic lung disease (CLD) of the newborn is characterized by prolonged respiratory disease requiring long-term care. The severity of CLD influences prognosis in premature infants. Originally, CLD was described as a disease of the small and terminal airways accompanied by fibrotic changes, but recently, the disease has been characterized by poor alveolar and capillary development. This change in our understanding of disease pathogenesis has promoted the development of therapies for respiratory distress syndrome, including exogenous artificial surfactants and non-invasive ventilation¹⁾²⁾. Because the etiology of CLD is multifactorial and has not been fully elucidated, no single effective treatment for CLD has been established.

Recent reports have shown that chorioamnionitis increases the risk of CLD³⁾⁴⁾⁵⁾. In these reports, the development of CLD was characterized by an early increase in inflammatory cells and mediators in infants affected by chorioamnionitis. Recently, we reported a positive relationship between the number of alveolar macrophages in the tracheobronchial aspirate fluid and the severity of lung injury in premature infants with chorioamnionitis⁶⁾. We concluded that alveolar macrophages in tracheobronchial aspirate fluid played a pathogenic role in CLD. Interleukin-6 (IL-6) has also been reported to be an important inflammatory mediator of CLD⁴⁾⁵⁾⁷⁾. This has been supported in an animal model demonstrating that systemic maternal inflammation induced by lipopolysaccharide (LPS) results in post-natal pulmonary inflammation and delayed alveolarization similar to CLD⁸⁾.

Alveolar macrophages play a critical role in the innate immune system of the alveolar space. They serve as primary phagocytes, clearing air spaces of infectious, toxic, or allergic particles, and regulate innate alveolar defense against respiratory infection⁹⁾.

When activated by large numbers of infectious particles or virulent microbes, alveolar macrophages synthesize and secrete a wide array of cytokines and chemokines⁹⁾. These cytokines and chemokines enact effective host responses to infection but can also contribute to tissue injury¹⁰⁾. Activation of alveolar macrophages contributes to the pathogenesis of various lung diseases, including acute lung injury, hypoxia, allergic inflammation, immune response to bacterial or fungal infections, and chronic heart failure¹¹⁾¹²⁾¹³⁾. Therefore, treatment of these diseases may benefit from regulating alveolar macrophage activation, particularly their secretion of cytokines and chemokines.

Acid sphingomyelinase (ASM) is a lysosomal enzyme that hydrolyzes sphingomyelin, a major lipid component of the plasma membranes, to ceramide and phosphocholine. Ceramide is a bioactive lipid that functions as second messenger in regulating cell proliferation, survival, and death. Over the past several decades, the sphingomyelin/ceramide signaling pathway has been implicated in the response to diverse cellular stressors, including radiation, infection, cytokines, death ligands, and cytotoxic agents¹⁴⁾. A previous report showed that macrophage ASM promoted cytokine secretion¹⁵⁾. In addition, ceramide production plays a role in several disorders, including diabetes, lung fibrosis, cardiovascular disease, multiple sclerosis, Alzheimer's disease, Wilson's disease, and depression¹⁶⁾. Therefore, regulating ASM may be a therapeutic tool for these disorders. Recently, functional inhibitors of acid sphingomyelinase (FIASMA) have been proposed as a large group of compounds that inhibit ASM and may be suitable for medical use in human¹⁷⁾.

The aim of the present study was to examine the relationship between ASM activation and lung inflammation and alveolar macrophages in a model of CLD. We

investigated the effects of maternal exposure to LPS on ASM activity in the infant rat lung and the effects of FIASMAs on IL-6 release from LPS-stimulated alveolar macrophages.

Materials and Methods

Animals

All experiments were performed in accordance with the approved institutional animal care guidelines of Akita University School of Medicine. Timed pregnant Wister rats were randomly assigned to two different treatment groups consisting of intra-peritoneal injection on fetal days 20 and 21 (term = 22 days) of either 3.5 mg/kg of LPS, O55:B5 (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) or the same volume of saline. Pregnant rats in each group were allowed to deliver at day 22. Until P1, pups were maintained in cages with dams, which were allowed to deliver on the same day as two groups without intra-peritoneal injection. Cage conditions were as follows: a constant temperature of $22 \pm 2^{\circ}\text{C}$, 12-h light/12-h dark cycle, and *ad libitum* access to food and water. On P1, pups were euthanized by ether overdose, and lungs were collected and processed.

Reagents

The following reagents were obtained from commercial sources: imipramine, chlorpromazine, maprotiline, nortriptyline, promethazine, doxepin, protriptyline (Sigma-Aldrich Co. LLC., St. Louis, MO, USA), and desipramine (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Cell culture and measurement of IL-6 release from macrophages

A rat alveolar macrophage cell line (ATCC8383, Rockville, MD, USA) was maintained in F-12K Nutrient Mixture (Life Technologies Corporation, Grand Island, NY, USA). Macrophages were plated into wells at a concentration of 1×10^5 cells/mL and stimulated with 1 μ g/mL of LPS, O55:B5 (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) for 2 h at 37°C with 5% CO₂ or with FIASMAs 1 h before LPS. In experiments using imipramine, cells were stimulated with LPS for 2–6 h. All FIASMAs excluding imipramine and chlorpromazine were added at a concentration of 10 μ M. Imipramine and chlorpromazine were added at a concentration of 30 μ M and 25 μ M, respectively. After stimulation with LPS, macrophages were separated from their media, and the cells and media were stored at -20°C. IL-6 levels in the culture media were measured using enzyme-linked immunosorbent assay (ELISA) kits (Pierce-Endogen, Rockford, IL, USA) according to the manufacturer's instructions.

Enzyme assay

Lungs were homogenized in CellLytic™ MT Cell Lysis Reagent (Sigma-Aldrich Co. LLC., St. Louis, MO, USA), and homogenates were centrifuged for 5 min at $10,000 \times g$ at 0°C. Supernatants were collected and stored at -30°C. Macrophages were lysed with water and disrupted by sonication on ice using three 15-s bursts. Lung-tissue supernatants and macrophage cellular homogenates were assayed for total protein by a BCA protein assay kit according to the manufacturer's protocol (Thermo Fisher Scientific Inc., Rockford, IL, USA) and for ASM activity as described below. Assays for cation-independent ASM and Zn²⁺-dependent secretory ASM activity were carried out as follows. ¹⁴C-labeled sphingomyelin was purchased from NEN Life Science Products. The standard 200- μ L assay mixture consisted of 100 μ L sample and 50 μ L assay buffer

containing 4% Triton X-100 (1.0 M sodium acetate, pH 5.0; final concentration of Triton X-100 in the 200- μ l assay mix, 1%). When added, the final concentrations of ethylene-diamine tetra-acetic acid (EDTA) and Zn^{2+} were 0.02 mM and 0.1 mM, respectively. The reaction was initiated by the addition of 50 μ L substrate (20 nmol, ^{14}C -labeled sphingomyelin, 0.08 μ Ci/20 nmol) in 0.2 % taurodeoxycholic acid. The assay mixtures were incubated at 37°C for 1 h and quantified as previously described ¹⁸). For lung tissues, the reactions were incubated for 6 h.

Western blots

Samples were lysed in RIPA buffer (Thermo Fisher Scientific Inc., Rockford, IL, USA) containing a protease inhibitor cocktail (Thermo Fisher Scientific Inc., Rockford, IL, USA). Samples were then sonicated, incubated on ice for 15 min, and centrifuged at 4°C for 15 min at $14,300 \times g$. Afterwards, the supernatants were collected. The protein concentrations of the cell extracts were quantified by a BCA protein assay kit according to the manufacturer's protocol (Thermo Fisher Scientific Inc., Rockford, IL, USA). Cell lysates were then diluted in an appropriate volume of NuPAGE LDS Sample Buffer that was supplemented with NuPAGE Reducing Agent (Life Technologies Corporation, Grand Island, NY, USA). Sample aliquots were heat-denatured for 10 min at 70°C. Equal amounts of protein (30 μ g) were separated by electrophoresis on a 10% NuPAGE Bis-Tris Gel System (Life Technologies Corporation, Grand Island, NY, USA) and were then transferred to a nitrocellulose membrane according to the manufacturer's instructions. The membrane was blocked in Pierce® Protein-Free T20 (TBS) Blocking Buffer (Life Technologies Corporation, Grand Island, NY, USA) for 1 h at room temperature. Subsequently, the membrane was incubated for 1.5 h at room temperature

with a primary rabbit polyclonal anti-ASM antibody (1:2,000) (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) and then for 1 h at room temperature with a secondary goat anti-rabbit IgG antibody that was coupled to horseradish peroxidase (HRP) (1:7,500) (Santa Cruz Biotechnology, Inc., Heidelberg, Germany). All antibodies were diluted in blocking buffer. The signal was visualized with an Immun-Star™ HRP Chemiluminescent Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions. The membrane was stripped and reprobed with a mouse monoclonal anti- β -actin antibody (Abcam plc, Cambridge, UK), which was used as an internal loading control.

Statistical analysis

The data were analyzed with Microsoft Office Excel 2012 software. $P < 0.05$ was considered statistically significant. Data are presented as means \pm standard error of the mean (SEM).

Results

ASM activity in the infant rat lung

First, ASM activity was measured in infant rat lung tissue after maternal LPS exposure using assay buffers with or without Zn^{2+} . ASM activity was measured with 2 different buffers (EDTA and Zn^{2+}) for each sample. ASM is activated by Zn^{2+} after its secretion from cells during conditions of oxidative stress or after cytokine stimulation. An increase in ASM activity with the addition of Zn^{2+} indicates that the cell has been exposed to an extracellular stimulus. Although maternal LPS exposure increased ASM

activity in the infant rat lung in both buffers (EDTA and Zn^{2+}), the effect was not statistically significant (Fig. 1).

IL-6 release from macrophages

We determined ASM activity of LPS-stimulated alveolar macrophages using assay buffers with or without Zn^{2+} . Unexpectedly, ASM activity in the alveolar macrophages was unaffected 1 h and 2 h after LPS stimulation (Fig.2). To investigate the onset of IL-6 production, IL-6 levels were measured in the media of alveolar macrophages 1 h or 2 h after the LPS stimulation (Fig.3). IL-6 levels significantly increased in the media of alveolar macrophages 2 h after LPS stimulation as compared with the media of alveolar macrophages without LPS stimulation. The increase in IL-6 was not apparent 1 h after LPS stimulation.

FIASMA and ASM activity in macrophages

To determine the effects of FIASMAs on alveolar macrophages, ASM activity was determined in LPS-stimulated alveolar macrophages treated with FIASMAs. The ASM inhibitors tested here were chlorpromazine, imipramine, maprotiline, nortriptyline, promethazine, doxepin, protriptyline, and desipramine (Table 1). Treatment with each FIASMA significantly reduced ASM activity of LPS-stimulated alveolar macrophages as compared with the cells without FIASMAs even with the addition of Zn^{2+} (Fig. 4). Basal ASM activities were not the same between experiments because of the cell conditions, but the reduction in ASM activity was generally 20% of the basal activity except in the case of doxepin, which reduced ASM to 30% of the basal activity (Fig. 5). Similar effects were observed with or without Zn^{2+} .

Western blots

We performed western blots to quantify ASM protein in LPS-stimulated alveolar macrophages. Representative results from western blots of macrophages treated with LPS plus FIASMAs are shown in Fig. 6. There was no difference in ASM protein level between samples treated with control solution or LPS and those treated with FIASMAs.

Effect of FIASMAs on IL-6 release from alveolar macrophage

To determine the effects of FIASMAs on IL-6 release from LPS-stimulated alveolar macrophages, we measured IL-6 levels in the media of alveolar macrophages 2 h after the LPS stimulation with the addition of FIASMAs. IL-6 levels in the media of the LPS-stimulated alveolar macrophages were significantly reduced by chlorpromazine, imipramine, maprotiline, doxepin, and protriptyline but not nortriptyline, promethazine, or desipramine (Fig.7). Basal levels of IL-6 in each experiment were not constant because of the cell conditions, and the levels of IL-6 were reduced to different extents by each FIASMA (40–80% of the basal level) (Fig. 8).

Next, we determined the level of IL-6 in the media of LPS-stimulated alveolar macrophages 2 h, 4 h, and 6 h after LPS stimulation in the presence of imipramine. IL-6 levels were significantly reduced 2 h and 4 h after stimulation with of LPS in the presence of imipramine. However, this reduction in IL-6 was not evident 6 h after stimulation (Fig. 9). ASM activity of LPS-stimulated macrophages remained low in the presence of imipramine 6 h after LPS stimulation (Fig. 10).

Discussion

A previous study reported that ASM activity was increased in the lungs of preterm sheep that received intra-amniotic LPS injection¹⁹⁾ The pregnant maternal sheep developed chorioamnionitis after intra-amniotic LPS injection, which caused secondary increases in ASM activity in the lungs of fetal sheep. Another study demonstrated that surfactant therapy in combination with imipramine, an ASM inhibitor, significantly improved acute lung injury induced by repeated airway lavage in the newborn piglet as compared with surfactant therapy alone²⁰⁾. These two reports suggested that the ASM/ceramide signaling pathway may be involved not only in neonatal lung injury but also in CLD of the newborn. In the present study, there was no significant increase in ASM activity in the lungs of newborn rats in which inflammation was triggered by maternal exposure to LPS. Our model induced inflammation with intra-peritoneal injection of LPS, which caused neonatal lung pathology via maternal systemic inflammation. In contrast, intra-amniotic injection of LPS into a pregnant rat might more directly promote fetal lung inflammation. This difference in methodology might be a reason that ASM activity was not significantly increased in our model of infant CLD.

Our results indicated that LPS stimulated the release of IL-6 from alveolar macrophages. However, ASM activity was not increased in LPS-stimulated alveolar macrophages. A previous study showed that LPS stimulation increased ASM activity and release of IL-6 from THP-1 macrophages²¹⁾. This difference may be due to inherent differences in cellular characteristics between rat alveolar macrophages and THP-1 macrophages.

By western blotting, we found no significant difference between samples treated with control solution or LPS and those treated with FIASMAs. Thus, ASM degradation did not occur in LPS-stimulated alveolar macrophages after a 2-h treatment with FIASMAs,

although the alveolar macrophages had decreased ASM activity. Weak organic bases, such as desipramine, can inhibit ASM activity¹⁷). ASM is thought to bind to intra-lysosomal membranes, protecting it from proteolytic inactivation²²). Desipramine and other FIASMAs cause ASM to detach from the intra-lysosomal membrane, leading to subsequent inactivation possibly by proteolytic degradation²²). However, our results suggested that degradation of ASM does not occur after a 2-h treatment with FIASMAs. This suggests that detachment of ASM from the intra-lysosomal membrane inhibits ASM activity even without proteolytic degradation.

It has been reported that one FIASMA decreased cytokine release from LPS-stimulated THP-1 macrophage²¹). In our study, some FIASMAs reduced the release of IL-6 from LPS-stimulated alveolar macrophages. However, this effect was present early and disappeared after a longer reaction time. Imipramine reduced the release of IL-6 from LPS-stimulated alveolar macrophages at 2 h or 4 h. This effect gradually diminished over 6 h, although ASM activity remained low. This suggested that ASM is involved in the early response to cytokine secretion from LPS-stimulated alveolar macrophage. Collectively, these results suggested that FIASMAs do not suppress IL-6 release from LPS-stimulated alveolar macrophage. The relationship between ASM activity and cytokine secretion by macrophages is controversial¹⁵). One review article suggested the mechanisms underlying ASM activation may determine whether it promotes or inhibits cytokine secretion¹⁵). Further studies are needed to elucidate the relationship between ASM activation and cytokine secretion from macrophages.

In conclusion, the present study was unable to confirm that ASM activation is responsible for LPS-related pathogenesis of CLD and IL-6 release from LPS-stimulated alveolar macrophages. Some FIASMAs, including imipramine, decreased IL-6 release

only at the beginning phase of the response. Further studies are needed to elucidate the relationship between ASM and infant lung inflammation in CLD.

Acknowledge

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Disclosures

The authors declare that there are no conflicts of interest.

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Legends for Figures

Fig. 1 The effects of maternal LPS exposure on ASM activity in lung tissues of d1 infant rats. ASM activity in infant rat lung tissues was determined using assay buffers with or without Zn^{2+} after maternal LPS exposure. The reactions were incubated for 6 h. Each bar represents the mean \pm SEM of 3 independent experiments.

Fig. 2 ASM activity of LPS-stimulated alveolar macrophages 2 h after stimulation. ASM activity of LPS-stimulated alveolar macrophages was determined using assay buffers with or without Zn^{2+} . Cells were incubated at 37°C for 0–2 h in F-12K Nutrient Mixture with LPS (1 μ g/mL) or the same amount of H_2O . Each bar represents the mean \pm SEM of 3 independent experiments.

Fig. 3 Release of IL-6 into the medium from LPS-stimulated alveolar macrophages. The levels of IL-6 in the medium of LPS-stimulated alveolar macrophages were determined using ELISA kits. Cells were incubated at 37°C for 0–2 h in F-12K Nutrient Mixture with LPS (1 μ g/mL) or the same amount of H_2O . Each bar represents the mean \pm SEM of 3 independent experiments.

Fig.4 The effects of FIASMAs on ASM activity of LPS-stimulated alveolar macrophages. ASM activity of LPS-stimulated alveolar macrophages was determined using assay buffers with or without Zn^{2+} after treatment with each FIASMA. Each bar represents

the mean \pm SEM of 3 independent experiments.

Fig.5 The percent decrease of ASM activities of LPS-stimulated alveolar macrophages after the treatment with each of FIASMAs. The percent decrease of the ASM activities were shown in the figure. The rates represent the mean \pm SE of three independent experiments.

Fig. 6 Western blotting of ASM protein in LPS-stimulated alveolar macrophages after treatment with each FIASM. Cells were incubated at 37°C for 0–2 h in F-12K Nutrient Mixture with LPS (1 μ g/mL) or the same amount of H₂O or in combination with LPS (1 μ g/mL) and a FIASMA. As a loading control, the membrane was probed with an anti- β -actin antibody

Fig. 7 Effects of FIASMs on IL-6 release from LPS-stimulated alveolar macrophages. IL-6 levels in the mediums of LPS-stimulated alveolar macrophages were significantly reduced by chlorpromazine, imipramine, maprotiline, doxepin, and protriptyline. IL-6 levels were not reduced by nortryptiline, promethazine, or desipramine. Each bar represents the mean \pm SEM of 3 independent experiments.

Fig. 8 Percent decrease in IL-6 release from LPS-stimulated alveolar macrophages after treatment with each FIASMA. The percentages represent the mean \pm SEM of 3 independent experiments.

Fig. 9 The effects of imipramine on IL-6 release from LPS-stimulated alveolar

macrophages 2–6 h after stimulation with LPS. Each bar represents the mean \pm SEM of 3 independent experiments.

Fig. 10 Effects of imipramine on ASM activity in LPS-stimulated alveolar macrophages 2–6 h after stimulation with LPS. Each bar represents the mean \pm SEM of 3 independent experiments.

Fig. 1

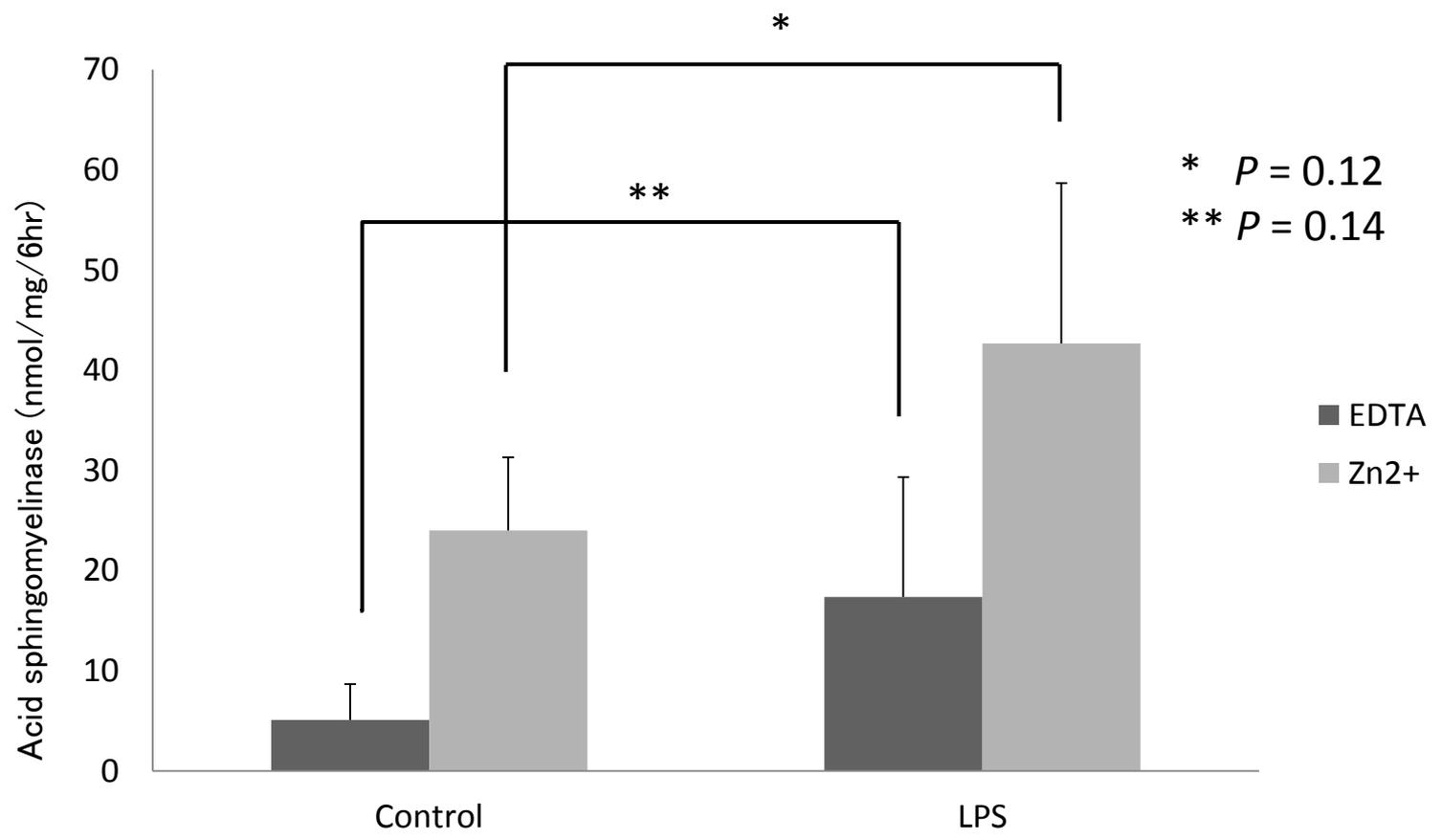


Fig. 2

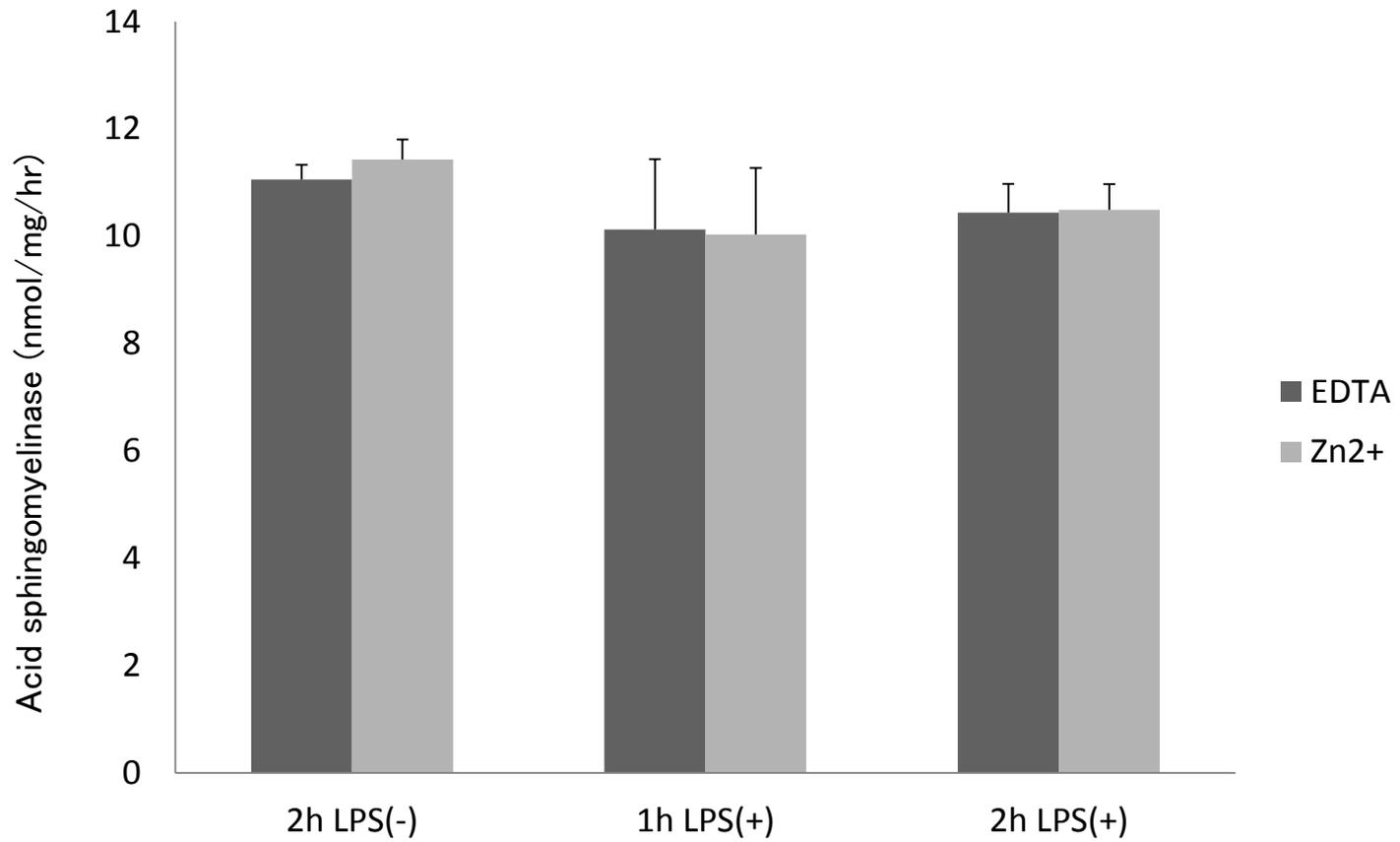


Fig. 3

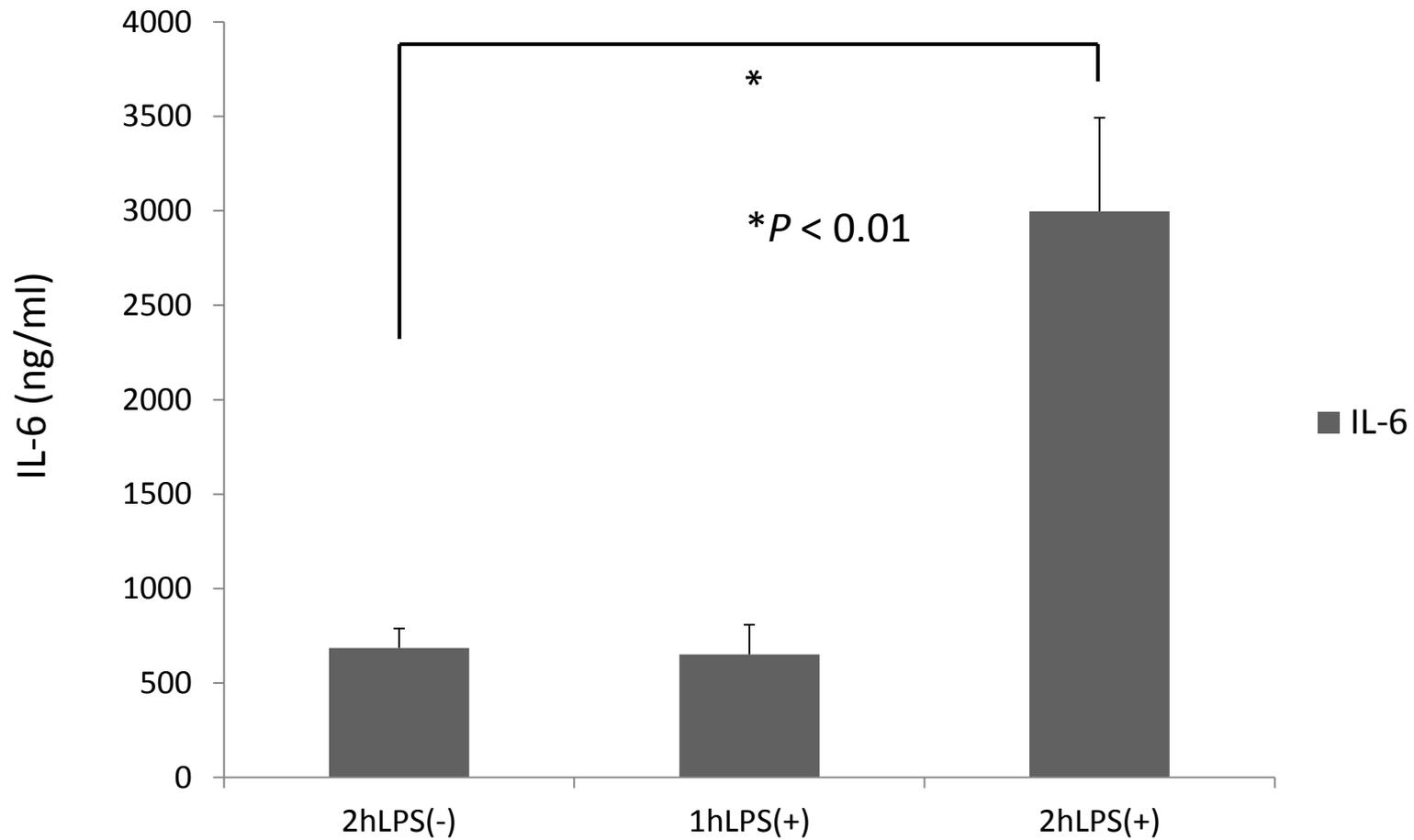


Fig. 4

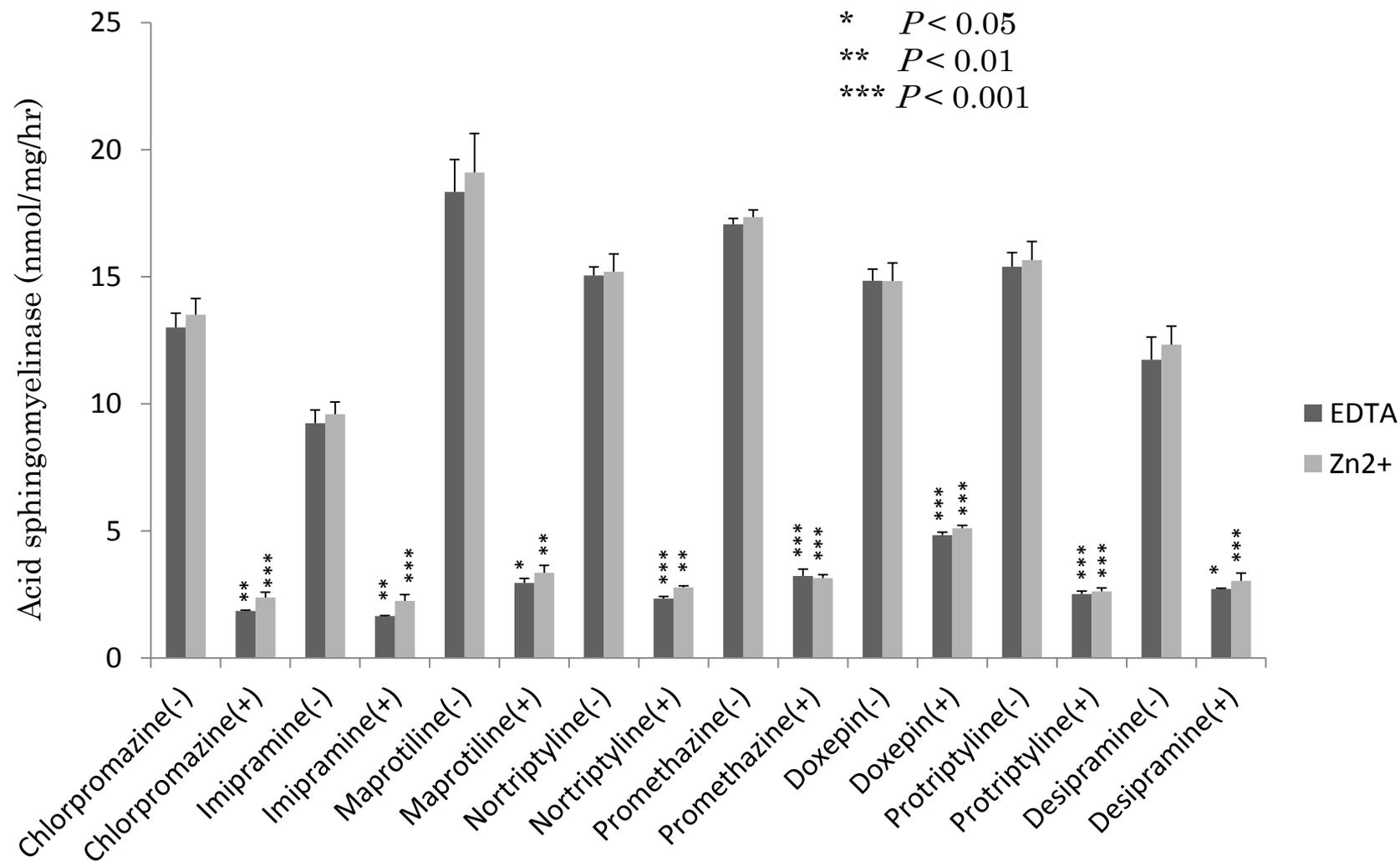


Fig.5

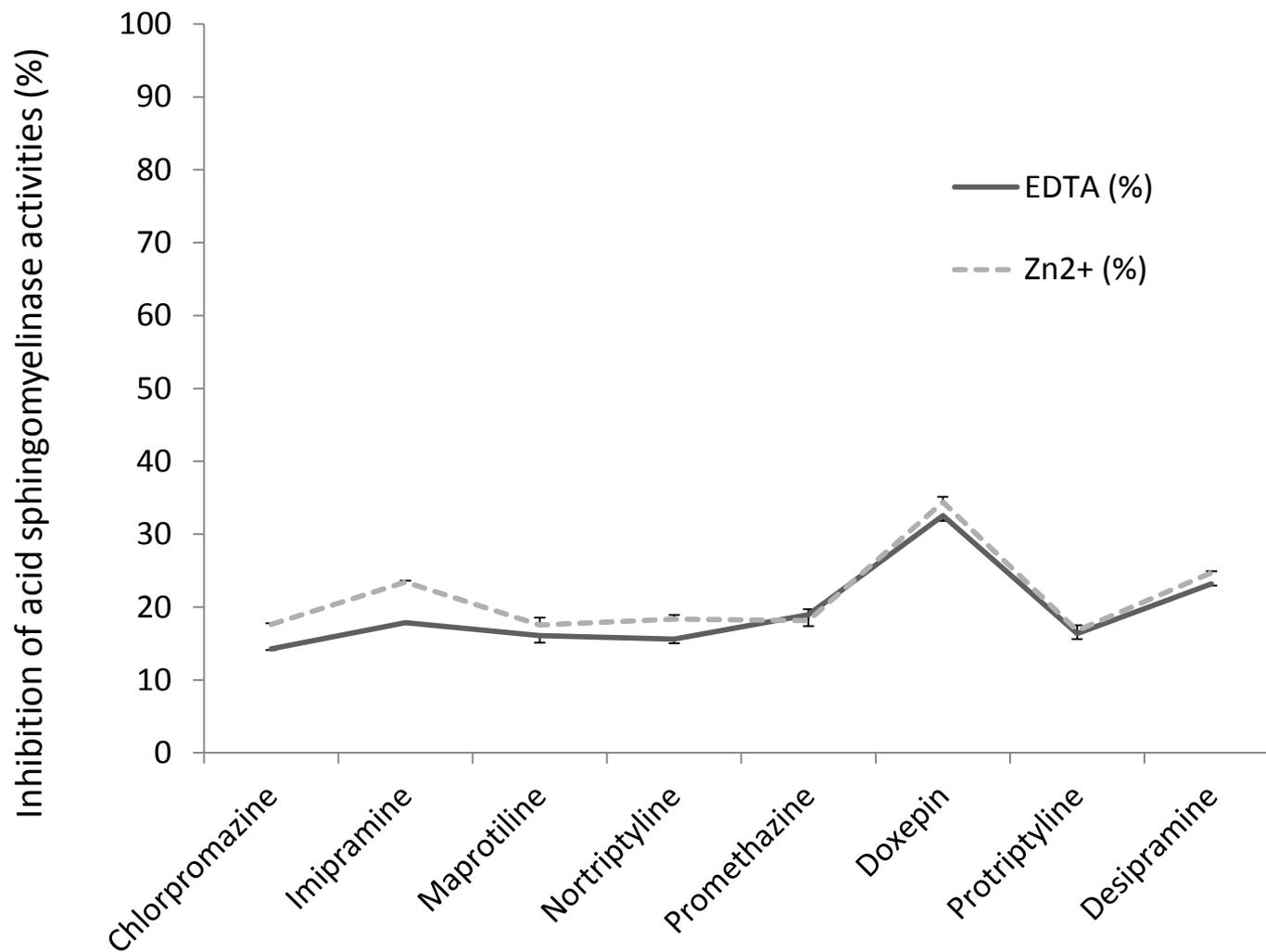


Fig. 6

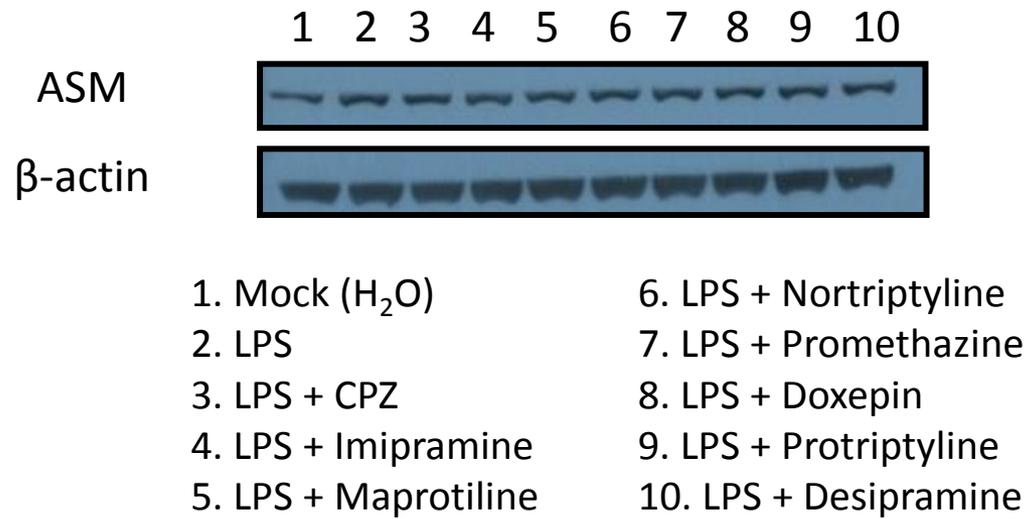


Fig. 7

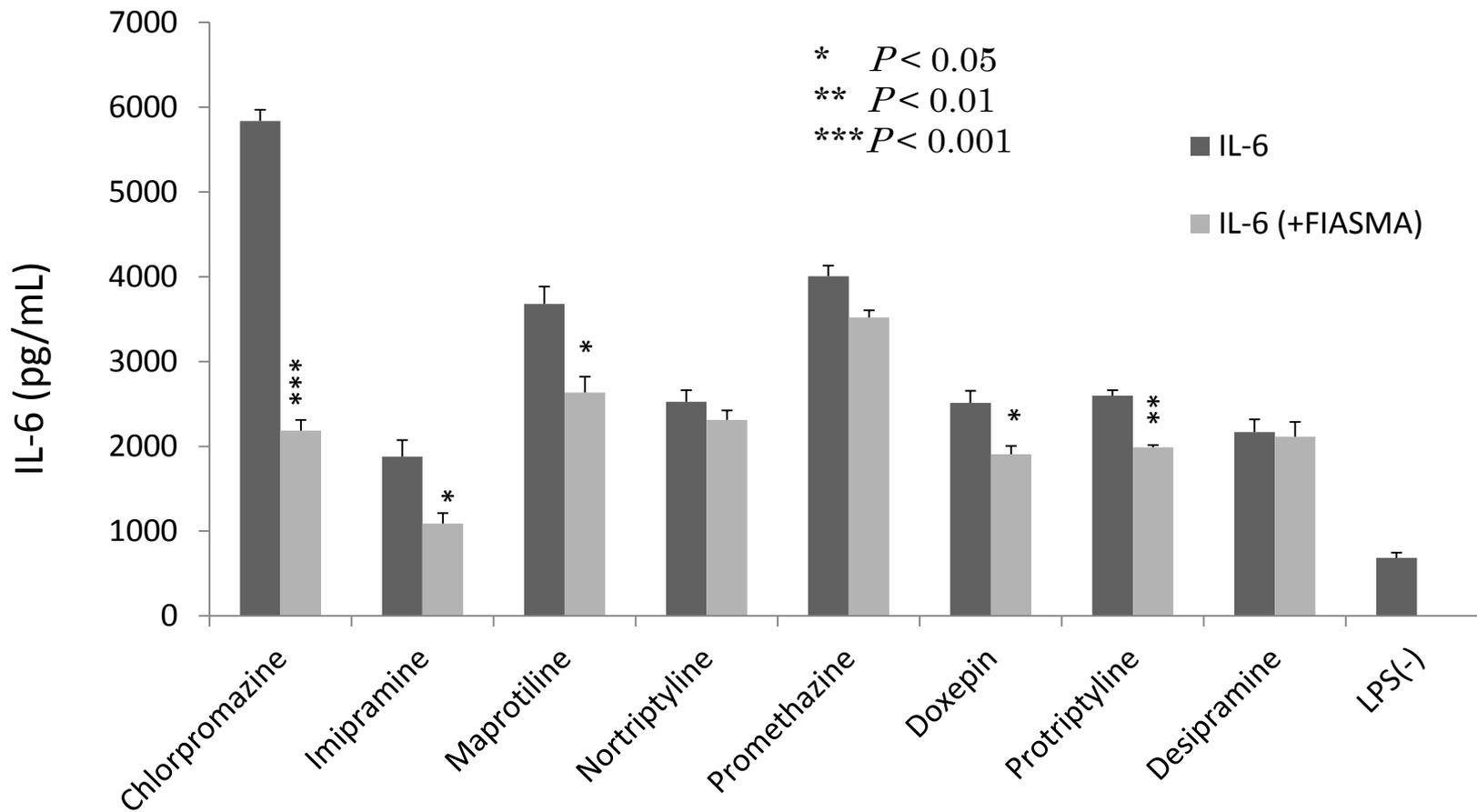


Fig. 8

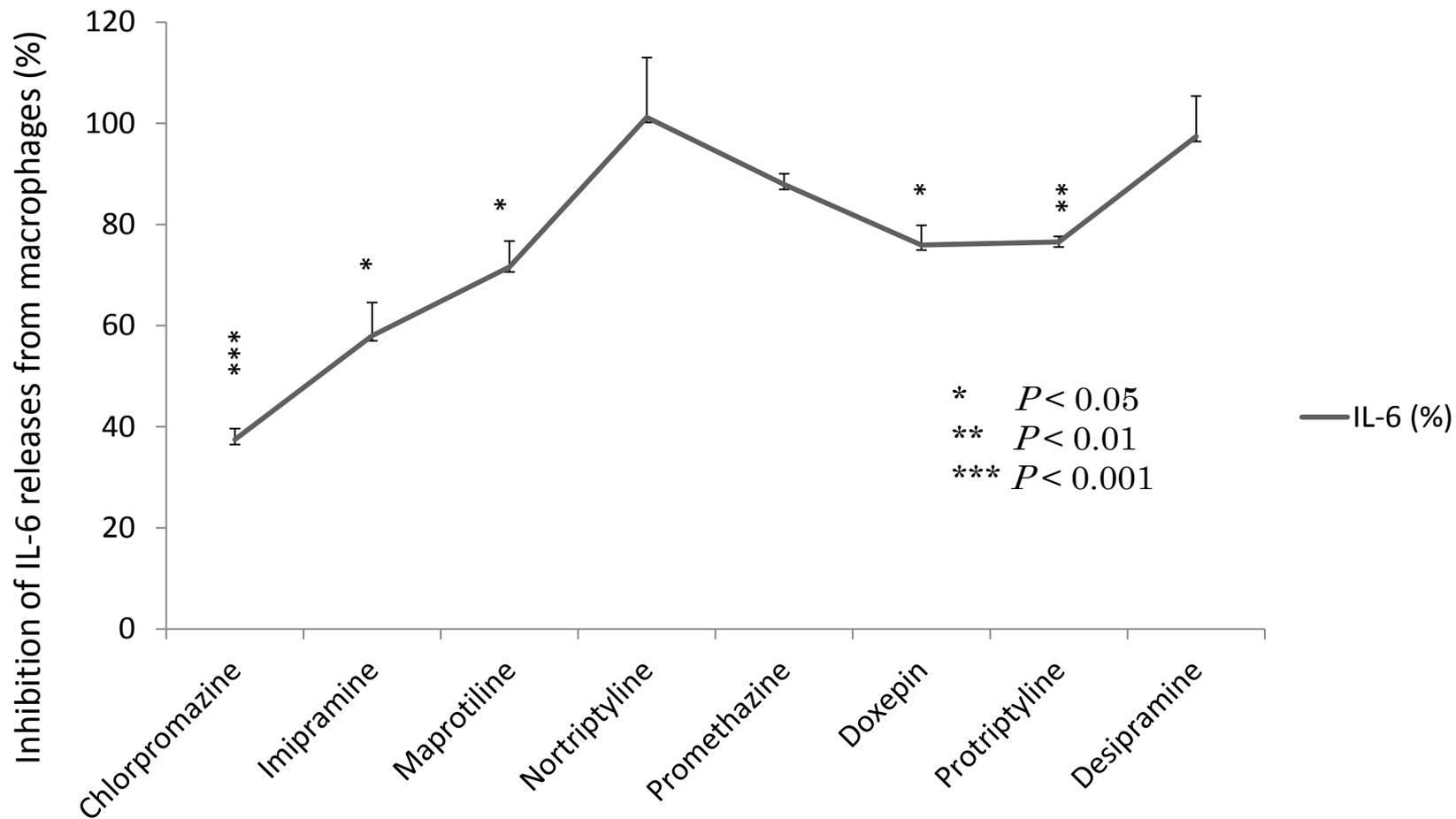


Fig.9

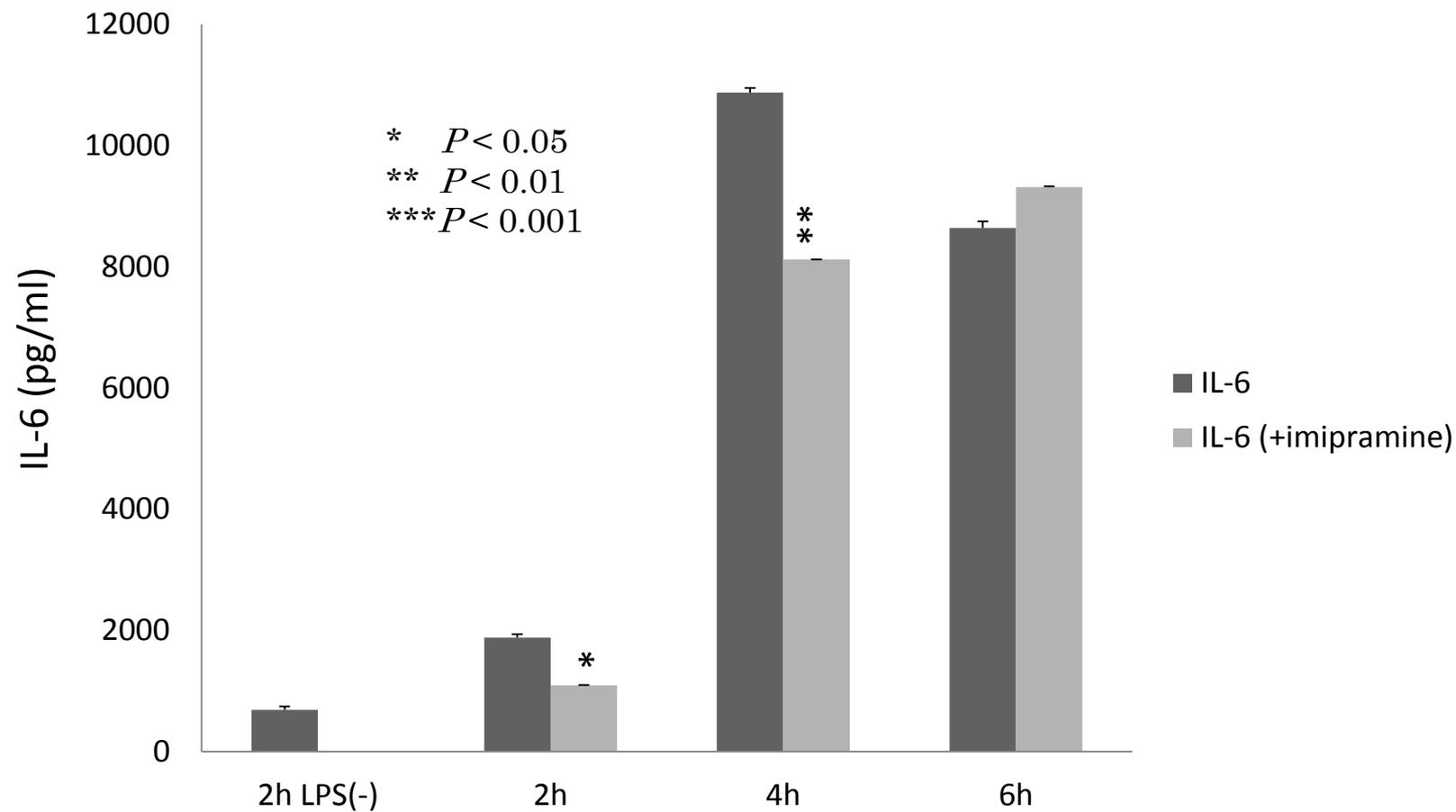


Fig.10

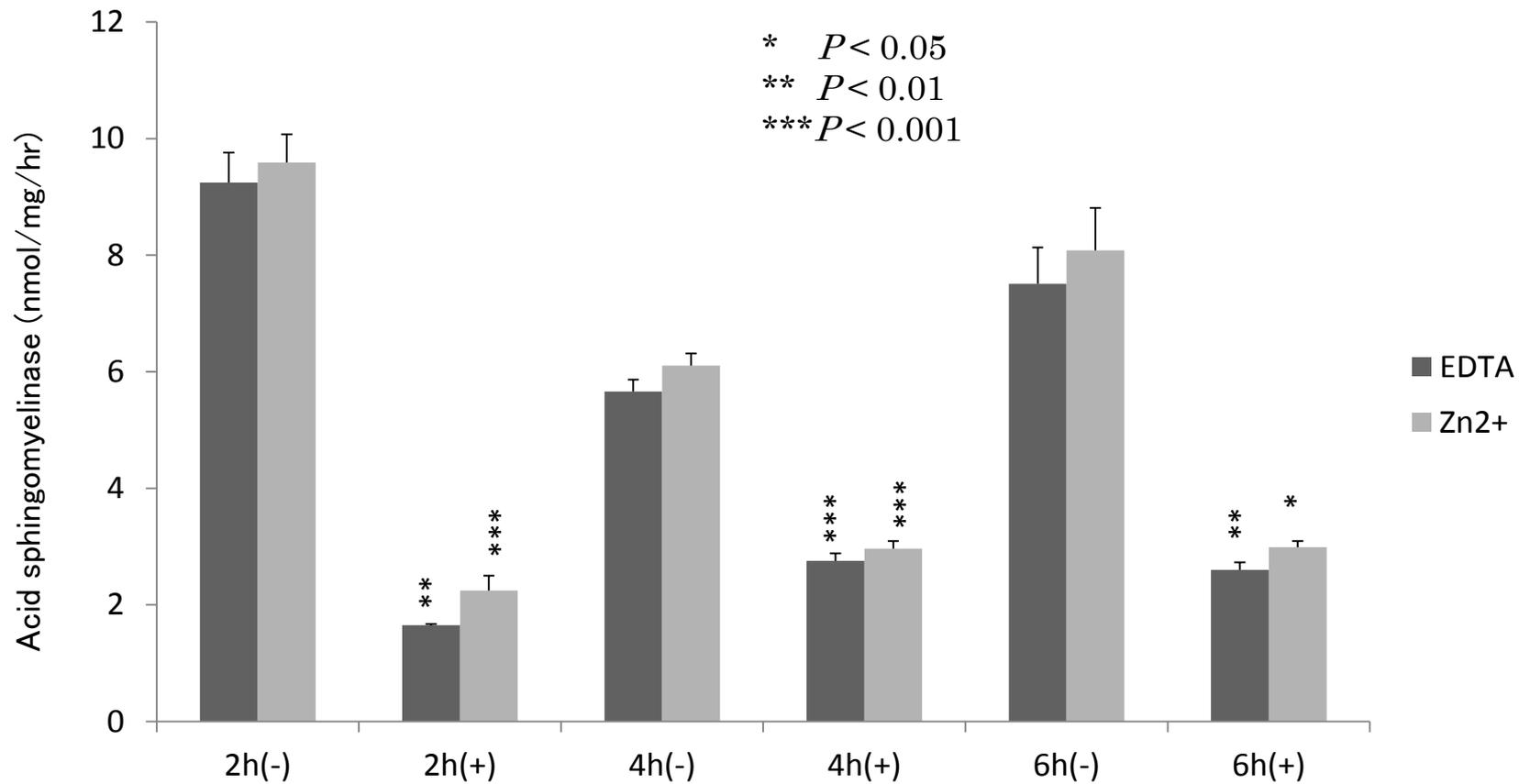


Table1 Functional inhibitors of acid sphingomyelinase (FIASMA) used in this study

FIASMA	Concentration (μ M)	Drug Class
Chlorpromazine	25	Antipsychotic
Imipramine	30	Tricyclic antidepressant
Maprotiline	10	Tricyclic antidepressant
Nortriptyline	10	Tricyclic antidepressant
Promethazine	10	Phenothiazine, antihistaminic
Doxepin	10	Tricyclic antidepressant
Protriptyline	10	Tricyclic antidepressant
Desipramine	10	Tricyclic antidepressant