Mol. Cells *27*, 251-255, February 28, 2009
DOI/10.1007/s10059-009-0049-4

Molecules and Cells

Inhibitory Effect of a Phosphatidyl Ethanolamine Derivative on LPS-Induced Sepsis

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Sepsis is the leading cause of death in critically ill patients. Today, around 60% of all cases of sepsis are caused by Gram-negative bacteria. The cell wall component lipopolysaccharide (LPS) is the main initiator of the cascade of cellular reactions in Gram-negative infections. The core receptors for LPS are toll-like receptor 4 (TLR4), MD-2 and CD14. Attempts have been made to antagonize the toxic effect of endotoxin using monoclonal antibodies against CD14 and synthetic lipopolysaccharides but there is as yet no effective treatment for septic syndrome. Here, we describe an inhibitory effect of a phosphatidylethanolamine derivative, PE-DTPA (phosphatidylethanolamine diethylenetriaminepentaacetate) on LPS recognition. PE-DTPA bound strongly to CD14 (K_d, 9.52 × 10^{-8} M). It dose dependently inhibited LPS-mediated activation of human myeloid cells, mouse macrophage cells and human whole blood as measured by the production of tumor necrosis factor-α (TNF-α) and nitric oxide, whereas other phospho-lipids including phosphatidylserine and phosphatidylethanolamine had little effect. PE-DTPA also inhibited transcription dependent on NF-κB activation when it was added together with LPS, and it rescued LPS-primed mice from septic death. These results suggest that PE-DTPA is a potent antagonist of LPS, and that it acts by competing for binding to CD14.

INTRODUCTION

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria and a well-known inducer of the innate immune response (Raetz and Whitfield, 2002; Ulevitch and Tobias, 1995). It consists of a hydrophobic lipid A component and the hydrophilic polysaccharides of the core and O-antigen. The cellular recognition of LPS is initiated by a cascade of three LPS receptors, LPS-binding protein (LBP), CD14 and the Toll-like receptor 4 (TLR4)/MD-2 complex (Triantafilou and Triantafilou, 2002). LBP is acutely induced in serum by infection and can extract LPS from the membranes of invading bacteria, or vesicles derived from them (Tobias et al., 1997). LPS bound to LBP is transferred to CD14 in monomeric form. It is ultimately delivered from CD14 to the TLR4-MD-2 complex and initiates intracellular signaling by promoting multimerization of the receptor complex.

CD14 is presented on the surface of myelomonocytic cells as a glycosylphosphatidylinositol (GPI)-linked glycoprotein, or in soluble form in the serum (Wright et al., 1990). In addition to the LPS of Gram-negative bacteria, other microbial products such as peptidoglycan (PGN), lipoteichoic acid, lipoarabinomannan and lipoproteins can be bound to CD14 (Dziarski et al., 1998; Gregory and Devitt, 1999). In addition to these microbial products, CD14 also has a marked affinity for cellular phospholipids. For example, anionic phosphatidylinositol (PI) can bind to CD14 and block LPS binding to CD14 *in vitro* (Wang et al., 1998). However, PI has only a moderate binding affinity for CD14, and it has not been shown to inhibit endotoxic activity in animal.

Engagement of LPS can cause a fatal septic syndrome if the inflammatory response is uncontrollably amplified. Sepsis is the leading cause of death in critically ill patients (Angus and Wax, 2001; Angus et al., 2001). Since there is as yet no effective treatment for septic syndrome, it will be important to identify antagonists of the LPS receptors. Previously, synthetic phospholipids with more than two acyl chains or oxidized phospholipids have been reported to block LPS signaling (Bochkov et al., 2002; Spyvee et al., 2005). However, phospholipid molecules mimicking physiological lipid structures have not displayed detectable LPS blocking activity in mouse models. In this paper, we show that a phospholipid derivative, phosphatidylethanolamine diethylenetriaminepentaacetate (PE-DTPA, Fig. 1A), with two myristoyl chains, can bind CD14 and block LPS recognition by its receptors. It also reduces endotoxemic symptoms in a mouse model.

MATERIALS AND METHODS

Reagents

LPS (*Escherichia coli*, serotype 0111:B4; trichloroacetic acid extracted) was purchased from List Biological Laboratories Inc. (USA). Phosphatidylethanolamine diethylenetriaminepentaacetate (PE-DTPA) was purchased from Avanti Polar Lipids, Inc. (USA). 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serin (PS), 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (PE) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (USA). Soluble form of mouse

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Received January 13, 2009; revised January 28, 2009; accepted January 28, 2009; published online February 20, 2009

Keywords: CD14, lipopolysaccharide, phospholipids, sepsis, TLR4

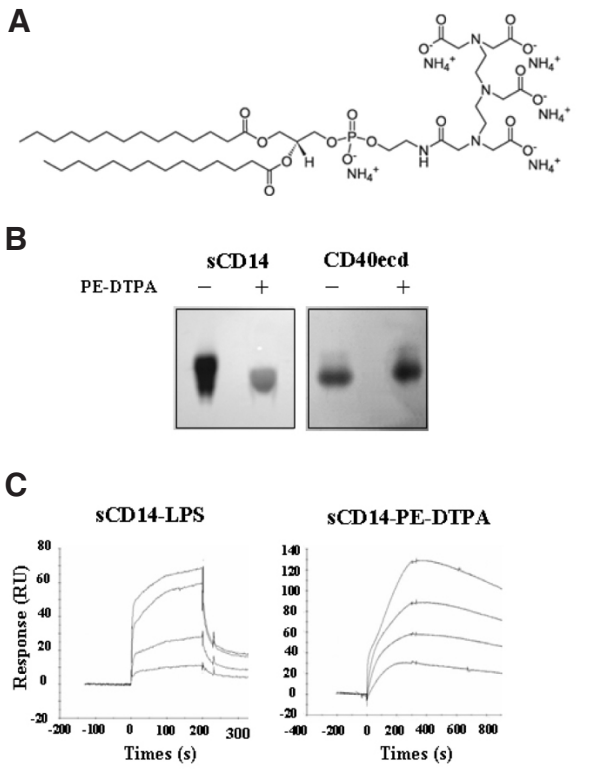

Fig. 1. PE-DTPA binds to CD14 with high affinity. (A) Chemical structure of PE-DTPA. (B) Affinity-purified mouse sCD14 and human CD40ecd were incubated with PE-DTPA in PBS at 37°C for 2 h. The molar ratio of protein to PE-DTPA was maintained at 1:10. After incubation, proteins were resolved by 8% native-PAGE and stained with Coomassie brilliant blue. (C) CD14 was immobilized on a CM5 Chip by amine coupling. The sensorgrams show the binding of ligand to immobilized CD14 versus time [expressed in RU] after subtraction of the control cell signal.

CD14 (sCD14) and ectodomain of human CD40 (CD40ecd) were kindly provided by Dr. Jie-Oh Lee's laboratory (Korea Advanced Institute of Science and Technology, Korea) (Kim et al., 2005).

Cell culture

RAW 264.7 macrophages and HEK 293 cells (American Type Culture Collection, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) inactivated fetal bovine serum (FBS) (Hyclone, USA) and antibiotic-antimycotic (GIBCO/BRL, USA). THP-1 cells (American Type Culture Collection, USA) were cultured in RPMI supplemented with 10% inactivated FBS. All cells were cultured at 37°C with 5% CO_2.

TNF-α ELISA assay

Cells were seeded in triplicate to a density of 1.2×10^4 cells/well in a 96-well plate, preincubated with phospholipids for 30 min and treated with 30 ng/ml LPS for additional 6 h at 37°C. After incubation, cell-free supernatants were collected and ELISA was performed using human TNF-α ELISA kit (BD Biosciences, USA) following manufacturer's instructions. Briefly, TNF-α capture antibody 1:250 diluted in 0.1 M sodium carbonate (pH 9.5) was used to coat 96 well plates (BD Falcon). Collected supernatant samples were then added to the wells and incubated for 2 h at room temperature. Bound TNF-α was detected with the serial addition of 1:250 diluted Detection antibody and Avidin-HRP reagent for 1 h at room temperature. Between each incubation step, unbound protein or antibody was washed off three times with PBS-0.05% Tween 20.

RT-PCR analysis

Total RNA was reverse transcribed with M-MLV Reverse Transcriptase (Promega, USA), according to the manufacturer's specification. Semi-quantitative PCR was performed using PCR Master Mix (Promega). The RT-PCR products were subjected to electrophoresis on agarose gel, and stained with ethidium bromide. For detection of human TNF-α mRNA, primers of sense, 5′-ATG AGC ACT GAA AGC ATG ATC CG-3′, antisense, 5′-TCA CAG GGC AAT GAT CCC AAA GT-3′ and for β-actin, sense, 5′-GTG GGG CGC CCC AGG CAC CA-3′, antisense, 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′ were used.

Nitrite assay

RAW 264.7 cells were pre-treated with phospholipids for 30 min and then stimulated with LPS for 24 h. Supernatants were collected for analysis of nitrites using the Griess reagent. Briefly, 100 μl mixture of 0.1% naphthylethylenediamine and 1% sulphanilamide were added to 100 μl of medium and incubated for 10 min at room temperature. The absorbance at 540 nm was determined using a Precision microplate reader (Molecular Devices, USA) with a standard curve constructed with nitrite solutions in the culture medium.

Cell viability assay

Cells were grown in triplicate to a density of 1.2×10^4 cells/well in a 96-well plate, treated as indicated in legends and incubated at 37°C. During the last 4 h of incubation, 50 μl of 1 mg/ml MTT was included in each well. After incubation, the MTT solution was discarded by aspiration, and the formazan produced by the viable cells dissolved in 80 μl dimethylsulfoxide. Absorbance was measured with a Precision microplate reader at 540 nm. Cell viabilities are expressed as percentages of the absorbance in the untreated control cells.

NF-κB Reporter activity

HEK 293 cells were seeded in 12-well plates at a density of 1.2×10^5 cells/well. Cells were transiently transfected with cDNA plasmids of mouse TLR4, MD-2 and CD14, pRL-SV (an internal control plasmid expressing the *Renilla* luciferase gene, Promega) and NF-κB reporter plasmid using Superfect (Qiagen GmbH, Germany) reagent. The cells were treated with LPS for 24 h and firefly and *Renilla* luciferase activities were measured sequentially in cell lysates using a Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's specifications by Victor3 (PerkinElmer LifeSciences, Great Shelford, UK). To normalize luciferase activities, relative luciferase activity is calculated as the ratio of the Firefly luciferase activity to *Renilla* luciferase activity.

Preparation and treatment of human whole blood

Blood was collected aseptically from 20- to 30-year old, normal male volunteers into sterile tubes containing EDTA. Aliquots of blood were added to 96-well plastic tissue culture plates and treated with phospholipids and LPS as indicated in legends. The plates were then incubated for 3 h at 37°C with 5% CO_2 on a shaking incubator. After incubation, the plates were centrifuged and plasma supernatants were saved for ELISA.

Surface Plasmon Resonance (SPR) analysis

Binding kinetics was assessed via SPR, using a BIAcore

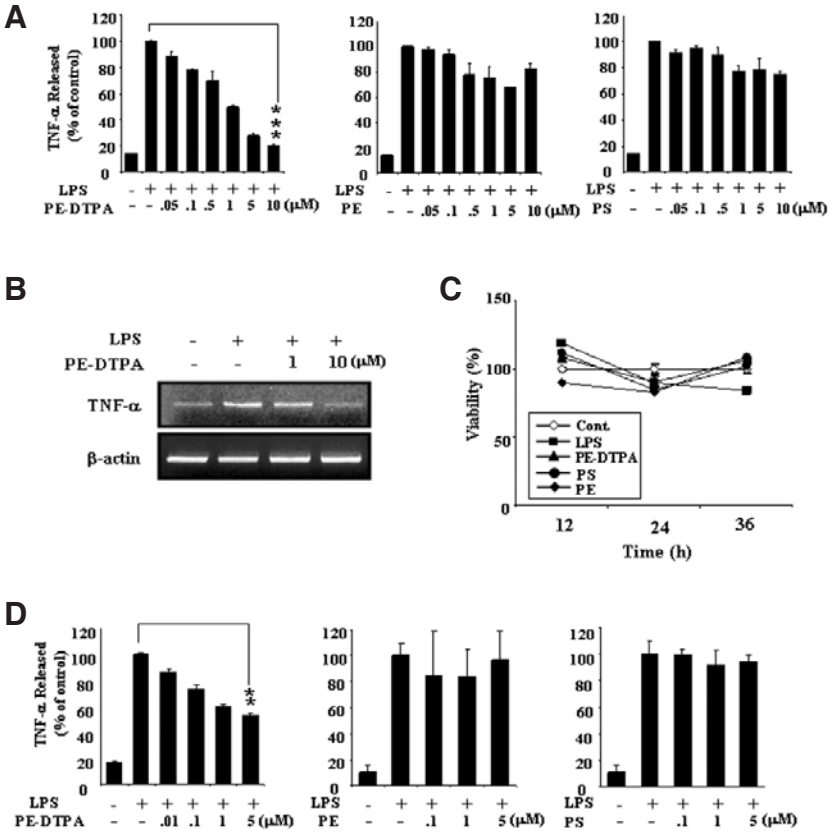

Fig. 2. Inhibition of LPS-induced TNF-α production by PE-DTPA in human myeloid cells and whole blood cells. (A) THP-1 cells were pre-treated with phospholipids at the indicated concentrations for 30 min and stimulated with 30 ng/ml LPS for 6 h at 37°C. Cell-free supernatants were collected and assayed for TNF-α by ELISA. No measurable TNF-α was observed in samples incubated without LPS. Data are means ± S.E. of triplicate experiments. Similar results were obtained in three independent experiments. (B) THP-1 cells were treated as in (A). After incubation, total RNA was analyzed by semi-quantitative RT-PCR. β-actin was used as an internal standard. (C) THP-1 cells were incubated with 0.5 μg/ml LPS, 10 μM PE-DTPA, 10 μM PS or 10 μM PE for the indicated times. Cell viability was evaluated by MTT assay and expressed as a percentage of the absorbance of untreated control cells. (D) Human whole blood was treated with 10 ng/ml LPS plus phospholipids at the indicated concentrations and incubated for 3 h at 37°C. After incubation, the whole blood was centrifuged and the resulting plasma supernatant samples were assayed for TNF-α by ELISA (**P < 0.1; ***P < 0.001).

3000™ biosensor instrument (BIACORE, Sweden). Briefly, CD14, at a concentration of 50 μg/ml in 10 mM sodium acetate buffer, pH 4.5, were immobilized to a CM5 sensor chip by amine coupling according to the manufacture's specifications. Un-reacted functional groups were blocked by the injection of 35 μl of 1 M ethanolamine-HCl, pH 8.0. Binding of LPS and PE-DTPA to the sensor chip was performed at a flow of 70 μl/min in 0.5 M NaCl/PBS. The sensor surfaces were regenerated by injecting 20 μl of 0.1% triton X-100 at the end of each cycle. The sensorgrams were corrected for signals in the reference flow cell and evaluated with BIAevaluation 3.0 software using a kinetic model. The apparent equilibrium dissociation constant, K_d, was calculated as the ratio of two kinetic constants (k_{off}/k_{on}).

In vivo studies

Eight- to 12-week-old male C57BL/6 mice were purchased from Daehan Biolink Co., LTD. (Korea). Mice were injected intraperitoneally with various amounts of PE-DTPA and 100 ng LPS plus 16 mg D-Gal in PBS. The survival of mice was counted for 3 d.

RESULTS

PE-DTPA binds to sCD14 with high affinity

We first determined whether PE-DTPA binds to sCD14 (soluble CD14). The native gel electrophoresis in Fig. 1B shows that pre-incubation of sCD14 with PE-DTPA led to a downward shift of the sCD14 band. PE-DTPA did not shift the CD40ecd (CD40 ectodomain) protein band, indicating that it binds selectively to sCD14. To measure the affinity between sCD14 and PE-DTPA and compare it with the affinity between sCD14 and LPS, we used SPR analysis. Affinity purified sCD14 was immobilized on a CM5 chip by amine coupling. The sensorgrams in Fig. 1C show the binding of 20-500 μg/ml LPS and 1-150 μM PE-DTPA to sCD14 (in RU), as a function of time. Equilibrium dissociation constants (K_d) were 1.3×10^{-5} and 9.5×10^{-8}, for LPS and PE-DTPA, respectively (Table 1). The strong affinity of PE-DTPA was due to its low dissociation rate. As a negative control, phosphatidyl ethanolamine and phosphatidyl serine were tested for binding, and both gave negligible binding.

Antagonistic effect of PE-DTPA on LPS-induced secretion of TNF-α and nitric oxide

The strong binding of PE-DTPA to sCD14 led us to see whether PE-DTPA inhibited the secretion of cytokine induced by LPS. In human myeloid THP-1 cells, LPS-induced secretion of TNF-α was inhibited in a dose-dependent manner by PE-DTPA (Fig. 2A). Secretion of TNF-α was reduced to basal level by 5-10 μM PE-DTPA. Although phosphatidyethanolamin (PE) and phosphatidylserine (PS) also caused some inhibition, it was less than 30% and was not dose-dependent. The IC_{50} of PE-DTPA for inhibition of LPS signaling was 0.75 ± 0.1 μM. Not only the level of TNF-α protein but also that of its mRNA was inhibited by pre-treatment with PE-DTPA, indicating that the latter inhibited transcriptional activation in response to LPS signaling (Fig. 2B). In a control experiment we showed that the viability of human myeloid THP-1 cells was not significantly affected by exposure to LPS, PE-DTPA, PS and PE for up to 36 h (Fig. 2C). Hence the reduced expression of TNF-α induced by PE-DTPA was clearly due to inhibition of LPS binding/signaling. We also examined the effect of PE-DTPA on freshly drawn human whole blood; PE-DTPA, but not PE and PS, inhibited LPS-induced TNF-α secretion (Fig. 2D).

It is known that LPS induces increased expression of induc-

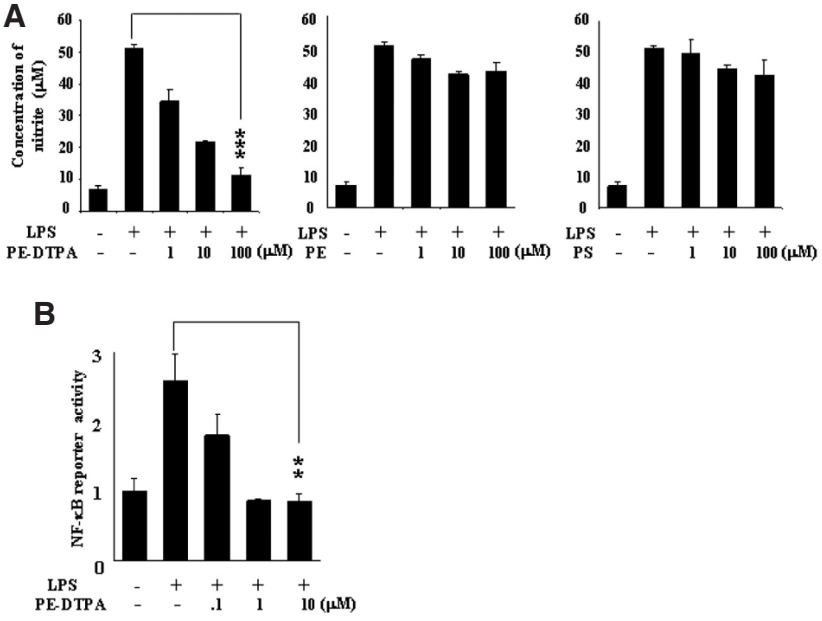

Fig. 3. Effects of PE-DTPA on nitric oxide release and NF-κB activation by LPS. (A) RAW 264.7 mouse macrophages were pre-treated with phospholipids at the indicated concentrations for 30 min and then stimulated with 0.5 μg/ml LPS for 24 h. Levels of nitrite in the culture medium were then measured. Data are means ± S.E. of triplicate experiments. (B) HEK293 cells were transiently transfected with cDNAs of mouse TLR4, MD-2 and CD14, and a luciferase reporter gene driven by an NF-κB dependent promoter. The cells were stimulated with 100 ng/ml LPS plus PE-DTPA at the indicated concentrations for 24 h and analyzed for luciferase activity. Values are presented relative to untreated control cells. This experiment is representative of three independent experiments (**P < 0.1; ***P < 0.001).

Table 1. Summary of equilibrium dissociation constants for the interaction of ligands with CD14.

	CD14			
Ligands	LPS	PE-DTPA	PE	PS
K_d (M)	1.33×10^{-5}	9.52×10^{-8}	NB[a]	NB

[a]Not binding

ible nitric oxide synthase (iNOS) and causes the release of endogenous nitric oxide in RAW 264.7 mouse macrophages. As shown in Fig. 3A, 0.5 μg/ml LPS stimulated secretion of nitric oxide from the macrophages more than 5-fold and this secretion was dose-dependently inhibited by pre-treatment with PE-DTPA. In contrast PE and PS had no effect.

To see whether PE-DTPA could inhibit LPS-induced TLR4-mediated NF-κB reporter activity, we transiently transfected HEK293 cells with plasmids of TLR4, MD-2 and CD14 together with reporter plasmids. LPS increased the NF-κB reporter activity of the transfected cells and PE-DTPA pre-treatment reduced reporter activity to basal level (Fig. 3B). Taken together, these data indicate that PE-DTPA antagonizes LPS binding and thus prevents intracellular signaling.

Antagonistic effect of PE-DTPA on LPS-induced mortality in mice

Since PE-DTPA bound to CD14 with high affinity and had a strong inhibitory effect on LPS-induced cytokine secretion in both human monocytes and mouse macrophages, we evaluated whether it could prevent LPS-induced sepsis in mice. Six C57BL/6 mice per group were peritoneally injected with 0.5 μg/kg LPS plus galactosamine and with various concentration of PE-DPTA. In the LPS-only injected group, 5 out of 6 mice died within 24 h (Fig. 4A). However, co-injection of as little as 2.5 mg/kg PE-DTPA resulted in the survival of half of the LPS-injected mice, and survival increased further with increasing concentrations of PE-DTPA. In the absence of PE-DTPA co-injection, but not in its presence, the livers of the LPS-injected mice were found to be severely hemorrhaged and to have undergone morphological changes (Fig. 4B). These results clearly demonstrate that PE-DTPA has a strong antagonistic effect on LPS-induced sepsis in mice.

DISCUSSION

We have obtained the following evidence that PE-DTPA is a powerful antagonist of LPS: (a) PE-DTPA bound to sCD14 with high affinity as shown by native-PAGE and SPR analysis (Fig. 1 and Table 1). (b) PE-DTPA pretreatment inhibited LPS-induced secretion of TNF-α and nitric oxide in human monocytes, human whole blood and mouse macrophages (Figs. 2 and 3). (c) PE-DTPA treatment reduced LPS-induced NF-κB dependent promoter activation (Fig. 3). (d) It also reduced the mortality of mice to LPS-primed sepsis (Fig. 4). The extremely high affinity of PE-DTPA to CD14 can be explained by the strong negative charge on the molecule. PE-DTPA contains five negatively charged groups, which is substantially more than physiological anionic phospholipids such as PI or PS. Consistent with our data, Wang et al. (1998) have shown that the number and position of negative charges in PI containing additional phosphate groups correlates with their binding affinities for CD14. Based on the crystallographic structure of CD14, Kim et al. (2005) proposed that the LPS binding pocket of CD14 contains several positively charged residues such as arginines and lysines near the opening of the pocket and that these groups interact with the negatively charged phosphate groups on the LPS. Thus, the negatively charged acetate and phosphate groups of PE-DTPA may interact with the positively charged residues at the opening area of the pocket while the lipid chains of PE-DTPA interact with the hydrophobic LPS binding pocket.

CD14 plays a role in pattern recognition of microbial products, especially in Gram-negative bacteria. Consistent with this, CD14 knock-out mice are resistant to shock induced by Gram-negative bacteria and LPS (Haziot et al., 1996). Although CD14 is constitutively expressed on many different cell types including monocytes, macrophages, neutrophils and epithelial cells, it is also an acute-phase protein that increases in response to inflammation and fatal septic shock (Bas et al., 2004). Therefore, it has been a target of potential antagonists of LPS recognition.

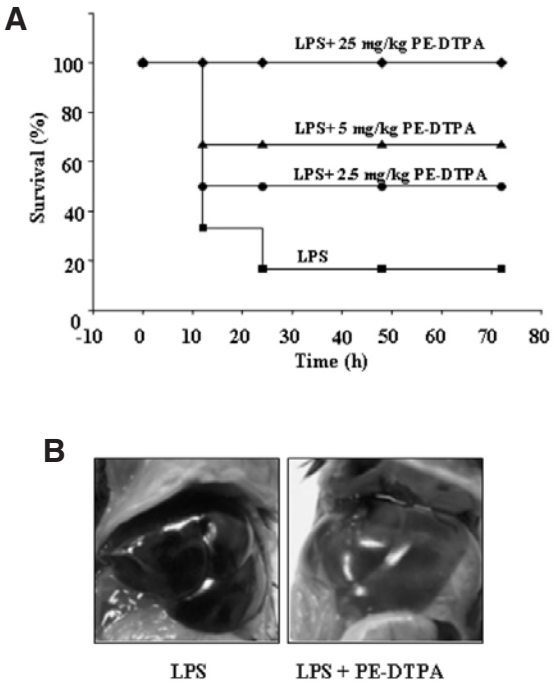

Fig. 4. Effects of PE-DTPA on mortality due to LPS treatment. (A) C57BL/6 mice were randomly grouped (6 mice per group). Vehicle on its own (■), 2.5 mg/kg PE-DTPA (●), 5 mg/kg (▲) or 25 mg/kg (◆) were intraperitoneally injected into the mice along with 500 ng/kg LPS and 80 mg/kg galactosamine. Mortality was observed for 72 h. (B) Left panel is the liver of mice injected with LPS and galactosamine at the same concentrations as above, showing severe hemorrhagic regions. The right panel shows the liver of a mouse that survived after being injected with LPS plus 5 mg/kg PE-DTPA.

In earlier studies, a monoclonal antibody against CD14 was found to inhibit LPS-CD14 binding and protect rabbits from the organ injury and lethality induced by LPS (Schimke et al., 1998). The anti-CD14 also attenuated acute lung injury in LPS-primed mice (Tasaka et al., 2003). Among phospholipids, PI and phosphorlyated forms of PI bound CD14 and blocked LPS-induced monocyte activation whereas PE, PC and phosphatidic acids had low binding affinities (Wang et al., 1998). The authors suggested that endogenous PI may modulate the down-stream responses resulting from cellular recognition of LPS. The present study about PE-DTPA is consistent with these previous reports. In addition, we showed that a synthetic derivative of phospholipid such as PE-DTPA had higher binding affinities for CD14 and were more effective antagonists of LPS recognition.

Several antagonists of TLR4-MD-2 have been developed for the treatment of septic syndrome. Among them, Eritoran (or E5564), a potent antagonist (Mullarkey et al., 2003; Rossignol and Lynn, 2005), is currently in a phase III clinical trial for septic syndrome. It is a synthetic lipid derived from the lipid A moiety of the non-pathogenic LPS of *Rhodobacter sphaeroides*. We have unpublished data showing that combined treatment with Eritoran and PE-DTPA results in better inhibition of LPS-induced TNF-α secretion in THP-1 cells than treatment with either compound on its own. Further development of LPS receptor antagonists is called to inhibit LPS recognition and to provide more effective treatment of septic syndrome.

ACKNOWLEDGMENTS

This work was supported by a Korea Science and Engineering Foundation grant funded by the Korean Government (Ministy of Education, Science and Technology) (R01-2007-000-11515-0) and the Brain Korea 21 Project (to H.-J.A. and H.L.).

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