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Glucose phosphorylated on carbon 6 suppresses lipopolysaccharide binding to lipopolysaccharide-binding protein and inhibits its bioactivities

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ABSTRACT

Lipid A comprises the active region of lipopolysaccharide (LPS), and its phosphate group is required for LPS activities. Additionally, it is essential for effects of inhibitors of LPS-induced coagulation activity in limulus amebocyte lysate (LAL) tests. Lipid A has phosphorylated glucosamine residues, which are structurally similar to glucose 1-phosphate (G1P) and glucose 6-phosphate (G6P). This study focused on the antagonistic effects of glucose phosphates on the action of protein or non-protein inhibitors against LAL coagulation, LPS–LPS-binding protein (LBP) interaction, and LPS bioactivities. These effects of glucose phosphates were evaluated and compared with those of other charged sugars such as fructose 6-phosphate and glucuronic acid by LAL tests, ELISA-based LPS–LBP binding assay, cell-based assay, and using a mouse endotoxin shock model. G6P neutralized the interfering actions of drug substances and plasma proteins on LPS coagulation activity in LAL tests. Compared to other sugars, G6P more strongly inhibited LPS binding to LBP, leading to significant inhibition of LPS-induced cellular responses in human umbilical vein endothelial cells and in the THP-1 human leukemic line. Consistent herewith, G6P inhibited inflammatory cytokine release and decreased serum alanine aminotransferase and hepatic caspase-3/7 activities and mortality in LPS-stimulated D-galactosamine-sensitized mice. These data indicated that the structural properties of G6P, such as its glucose moiety and phosphorylation on carbon 6, are important for suppressing the interaction of proteins with LPS. Therefore, G6P is useful to improve sensitivity and accuracy of plasma and drug LPS assays, and such structural property is more suitable to antagonize LPS activities.

1. Introduction

The endotoxin lipopolysaccharide (LPS) is a component of bacterial cell walls, which induces potent pathophysiological effects, including gram-negative bacterial sepsis, in mammals [1]. The active center of LPS has two conjugated glucosamine residues attached to varying numbers of long-chain fatty acids, with one phosphate group on each carbohydrate [2,3]. This distinctive lipid A structure is essential for LPS bioactivity; the omission of one phosphate group significantly reduces endotoxicity of LPS, whereas alterations in the monosaccharide structure have little effect [4].

LPS-treated monocytes and macrophages secrete a wide array of inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6 [5]. LPS induces the expression of intercellular cell adhesion molecule-1 and the endothelial adhesion molecule *E*-selectin [6,7]. Circulating LPS is recognized by LPS-binding protein (LBP) [8], which transfers LPS to its receptor CD14 [9] and to TLR-4/MD-2 complexes [10]. LBP has a cluster of cationic N-terminal residues essential for LPS binding [8]. In addition, the negative charge and hydrophobic interactions of the phosphate groups and fatty acids of lipid A are important for the interaction of LPS with LBP and CD14. After binding of LPS to the TLR-4/MD-2 complex, TLR-4 dimerizes, leading to the activation of intracellular signaling molecules such as nuclear factor-κB, mitogen-activated protein kinases, and interferon response factor 3 [11]. This results in increased expression of numerous genes encoding cytokines, chemokines, and type I interferon (IFN). Therefore, severe sepsis has been associated with imbalances of pro- and anti-

Abbreviations: BETs, bacterial endotoxin tests; BSA, bovine serum albumin; CD, cluster of differentiation; CPFX, ciprofloxacin; CSE, control standard endotoxin; EGM2, endothelial cell growth media-2; ELISA, enzyme-linked immunosorbent assay; EU, endotoxin units; F6P, fructose 6-phosphate; $FeSO_4$, iron sulfate; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; D-GalN, D-galactosamine; GlcUA, glucuronic acid; HUVECs, human umbilical vein endothelial cells; IL, interleukin; INF, interferon; LAL, limulus amebocyte lysate; LBP, LPS-binding protein; LPS, lipopolysaccharide; MD, Myeloid differentiation factor; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; RPMI, Roswell Park Memorial Institute; TLR, toll-like receptor; TNF, tumor necrosis factor.

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inflammatory cytokines in LPS-activated monocytes and macrophages [12]. Based on this, inhibitors of LPS interactions with receptors and TLR-4 signaling systems, including LPS antagonists and receptor-specific antibodies, have been investigated as treatments for endotoxemia [13]. Among these, the lipid A precursor IVa (synthetic compound 406) [14] and the phospholipids phosphatidylinositol and cardiolipin [15] showed antagonistic activities against LPS. However, no effective therapy against endotoxemia has been identified, reflecting the complexity of the pathways involved in severe endotoxic shock and the limited therapeutic effects of inhibitors of inflammatory cytokines when solely administered. Furthermore, eritoran, a lipid A analog, in which the acyl group was exchanged for an alkyl group, was developed as a TLR-4 antagonist for the treatment of severe sepsis; however, eritoran did not achieve improvement in the primary outcome measure of 28-day all-cause mortality in a phase III study.

Coagulation reactions in the amebocyte lysates of horseshoe crabs have been used for decades to detect trace concentrations of LPS. Such reactions are the basis of limulus amebocyte lysate (LAL) tests to detect LPS contaminants in injections administered to humans and animals [16]. These methods have been adopted as standard bacterial endotoxin tests (BETs), described in the United States Pharmacopeia [17]. Multiple severe responses are induced by LPS-contaminated injections, highlighting the need for accurate and sensitive assays for LPS to ensure the safety of injection solutions. During clinical endotoxemia or septic shock, circulating LPS induce a strong pathophysiological response, and LAL tests are widely used to detect LPS levels in patient blood samples in such circumstances [18]. However, these tests are susceptible to interference by various protein and non-protein substances that inhibit the coagulation activity of LPS, hindering accurate determination of endotoxin levels in drug solutions and plasma [19,20]. In a recent study, monophosphoryl lipid A (the dephosphorylated form of lipid A) showed low sensitivity to the interfering actions of iron sulfate ($FeSO_4$) as compared to lipid A [21]. This suggested that the phosphate group of lipid A is important for the action of substances that interfere with LPS coagulation. Further, this finding indicated that analogs of the phosphorylated glucosamine in lipid A might inhibit interactions of lipid A with other mediators of LPS activity. Therefore, in this study, we evaluated the glucose phosphates glucose-1-phosphate (G1P) and glucose-6-phosphate (G6P) (PubChem CID65533 and CID5958, respectively) as partial analogs of lipid A for desirable properties in LAL tests [22].

G1P and G6P have glucose moieties phosphorylated on carbons 1 and 6, respectively, each of which has a negative charge. Several studies have described antagonistic effects of lipid A or LPS analogs on LPS–LBP binding and LPS bioactivities [23]; however, there are no reports on the correlations between the structural properties of glucose phosphates and their antagonistic effects on LPS activities. Thus, to evaluate whether glucose phosphates might improve the sensitivity and accuracy of BETs for injectables or the clinical diagnosis of endotoxemia, we initially investigated their effects in LAL tests. Next, we investigated their antagonism of LPS bioactivities and compared there in vitro effects with those of fructose 6-phosphate (F6P) and glucuronic acid (GlcUA) as examples of other charged sugars. Finally, we evaluated the therapeutic effects of G1P and G6P in vivo, using D-galactosamine-sensitized mice.

2. Materials and methods

2.1. Materials

Purified LPS from *Escherichia coli* O55:B5, fructose 6-phosphate (F6P) disodium salt, D-glucuronic acid (GlcUA) sodium salt, and D-galactosamine (D-GalN) were from Sigma-Aldrich (St. Louis, MO, USA). α-D-Glucose-1-phosphate (G1P) disodium salt, D-glucose-6-phosphate (G6P) monosodium salt, D(+)-glucose, ascorbic acid sodium salt, phosphoric acid, ciprofloxacin (CPFX), phosphate buffer, and phosphate-buffered saline (PBS) were from Wako Pure Chemical Industries (Osaka, Japan). Iron sulfate heptahydrate was from Nacalai Tesque (Kyoto, Japan). Zymosan was from InvivoGen (San Diego, CA, USA). Epirubicin hydrochloride was from Sawai Pharmaceutical Co. (Osaka, Japan). Human umbilical vein endothelial cells (HUVECs) were from Lonza Japan (Tokyo, Japan) and THP-1 (TIB-202) cells were from the American Type Culture Collection (Manassas, VA, USA). Phorbol 12-myristate 13-acetate (PMA), used to induce the differentiation of THP-1 cells, was from Wako Pure Chemical Industries. CellTiter96 Aqueous One Solution cell proliferation assays for 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) tests were from Promega (Madison, MI, USA). All test compound solutions were adjusted to pH 7–7.5, using hydrochloric acid (HCl) or sodium hydroxide (NaOH).

2.2. LAL test

LAL coagulation activity was measured by the Limulus ES-2 Test, a kinetic turbidimetric method, with control standard endotoxin (CSE) from *E. coli* UKT-B (Wako Pure Chemical Industries). The CSE solution was diluted to 0.1, 0.05, and 0.025 endotoxin units (EU)/mL for standard solutions. To assess the inhibitory effects of G1P and G6P on the interfering actions of 0.1 mM $FeSO_4$, 0.5 mM CPFX, and 0.025 mg/mL epirubicin solutions, sample solutions were prepared in the absence or presence of 0.625, 1.25, 2.5, 5, and 10 mM G1P or G6P, 10 mM glucose, or 10 mM phosphate buffer; these concentrations do not interfere with LAL coagulation activity. All test compounds were dissolved and diluted in water. After the addition of 0.05 EU/mL CSE to the sample solutions, the turbidity was measured using the Toxinometer ET-6000/J (Wako Pure Chemical Industries), as previously reported [21]. The LPS concentration in sample solutions was calculated according to LAL coagulation times using a standard curve generated with CSE standard solutions; the recovery of LPS was calculated as follows: recovery (%) = measured LPS concentration (EU/mL)/spiked LPS concentration (EU/mL) × 100. To determine LPS in heparinized rat plasma, CSE solutions were diluted to 0.01, 0.0025, and 0.000625 EU/mL. Blood was collected from four male Sprague-Dawley rats (SLC Inc., Shizuoka, Japan) and plasma was collected by centrifugation after the addition of heparin individually. Next, 0.025 EU/mL LPS was spiked into the plasma samples, which were then diluted up to 10-fold in 0.5, 1, 1.5, 2, and 2.5 mM G1P or G6P, 2.5 mM F6P, GlcUA or glucose solutions in water or in 2.5 mM phosphate buffer and heated at 75 °C for 10 min. As negative controls, the LAL activities of water, $FeSO_4$, CPFX, epirubicin, and plasma solutions without CSE were measured.

2.3. Enzyme-linked immunosorbent assay-based LPS–LBP-binding assay

Maxisorp 96-well plates (Nunc, Minneapolis, MN, USA) were coated with 100 ng *E. coli* O55:B5 LPS diluted in pyrogen-free PBS and plates were sealed and incubated overnight at 4 °C. Then, the wells were washed three times with wash solution (PBS containing 0.03% Tween-20; 200 μL/well) and unoccupied sites were blocked by incubation in PBS containing 0.5% low-endotoxin bovine serum albumin (BSA, Nacalai Tesque) for 2 h at room temperature. The blocking solution was subsequently removed and wells were washed as described above. G1P or G6P (at 12.5, 25, or 50 mM) or 50 mM F6P, GlcUA, glucose, or ascorbic acid was added at a volume of 50 μL/well to wells coated with immobilized LPS. All test compound solutions were diluted using PBS containing 0.5% low-endotoxin BSA. Next, 50 μL 200 ng/mL recombinant human LBP (R&D Systems, Minneapolis, MN, USA) was added to each well (final concentrations of G1P and G6P: 6.25, 12.5, or 25 mM, other test compounds: 25 mM, LBP: 100 ng/mL). In order to reveal the differences between the inhibitory effect of G6P and that of other sugars, we compared them at a concentration of 25 mM, at which G6P but not G1P showed a significant effect. After mixing, the plate was incubated at room temperature for 2 h and bound LBP was detected after an initial incubation with anti-human LBP mouse monoclonal antibody (LifeSpan