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Lipid A 3′-*O*-deacylation by *Salmonella* outer membrane enzyme LpxR modulates the ability of lipid A to stimulate Toll-like receptor 4

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ABSTRACT

Modification of lipopolysaccharides, including the membrane anchor portion lipid A, is essential for bacterial adaptation to its host. We examined whether lipid A 3′-*O*-deacylation by *Salmonella* lipid A deacylase LpxR affected the ability of lipid A to stimulate the Toll-like receptor 4 (TLR4) and MD-2 complex. Unmodified lipid A and 3′-*O*-deacylated lipid A were purified from *Escherichia coli* and *E. coli* expressing recombinant LpxR, respectively. Inactive lipid A species, palmitoylated lipid A and a lipid A biosynthetic precursor lacking the myristate moiety were purified from *E. coli* expressing recombinant *Salmonella* lipid A palmitoyltransferase PagP and *E. coli* mutant defective in lipid A biosynthesis, respectively. Mass spectrometric analysis of the purified lipid A preparations showed a spectra of single lipid A species and gave a single band on thin layer chromatography. An NF-κB-dependent reporter activation assay was used to determine the bioactivity of the lipid A species in a cell line that expressed human TLR4 and MD-2 complex. Deacylated lipid A was less active than unmodified lipid A, suggesting that lipid A 3′-*O*-deacylation by LpxR is beneficial for bacteria to evade host immune surveillance: On the other hand, deacylated lipid A was more active than palmitoylated lipid A and the lipid A precursor. Taken together, these results indicated that lipid A 3′-*O*-deacylation by LpxR significantly reduces the bioactivity of lipid A.

1. Introduction

Innate immune responses are induced by an array of microbial components including the endotoxin lipopolysaccharide (LPS), which is the major component of the gram-negative bacterial outer membrane [1,2]. Excessive cellular responses against LPS can induce sepsis, which is a leading cause of mortality among severely ill patients with infectious diseases [2,5]. The bioactive component of LPS is its membrane anchor portion lipid A. The precise structure of lipid A is recognized by an innate immune receptor on the cell surface, Toll-like receptor 4 (TLR4) and MD-2 complex [3]. Dimerization of the TLR4-MD-2 complex is induced by an association with lipid A, which in turn initiates cellular innate immune responses, such as the production of pro-inflammatory cytokines, adhesive proteins, bioactive lipids, and reactive oxygen species [3,4].

The structure of lipid A is relatively conserved among different enteric bacteria. The lipid A domain of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is a β-1′,6-linked disaccharide of glucosamine, which is phosphorylated at the 1 and 4′ positions and acylated at the 2, 3, 2′ and 3′ positions with 3-hydroxymyristate. The hydroxyl groups of the 3-hydroxymyristate attached at positions 2′ and 3′ are further acylated with laurate and myristate, respectively (Fig. 1) [1,2]. The hexa-acylated lipid A is coupled to a core oligosaccharide at the 6′ position and assembled with O-antigen to form the mature LPS molecule in the bacterial inner membrane, which is then transported to the outer membrane [1,2]. Previous studies have demonstrated the importance of the conserved hexa-acylated structure of lipid A for its endotoxin activity. LPS that was prepared from a lipid A biosynthetic mutant lacking LpxM, which catalyzes the attachment of myristate moiety at hydroxyl group of 3-hydroxymyristate attached at 3′ position of lipid A (Fig. 1), showed a dramatically reduced induction of cytokines from human macrophage-like cell lines and stimulation of E-selectin expression in human endothelial cells [6,7]. Moreover, monophosphoryl lipid A, a derivative that lacks the phosphate residue attached at the 1-position, was able to stimulate some innate immune responses albeit to a lesser extent than toxic lipid A [8,9]. Therefore, monophosphoryl lipid A and its derivatives have been used as potent adjuvants [10].

In many pathogenic bacteria LPS is further modified by bacterial enzymes in response to environmental conditions, including host tissues, and these modifications are essential for bacterial adaptation to its host [11]. In *S.* Typhimurium, the acyl chain moi-

Abbreviations: LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; *S.* Typhimurium, *Salmonella enterica* serovar Typhimurium; TLC, thin layer chromatography; HPTLC, high performance thin layer chromatography; U-lipid A, unmodified lipid A; P-lipid A, palmitoylated lipid A; R-lipid A, 3′-*O*-deacylated lipid A; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; M-lipid A, lipid A precursor lacking myristoyl fatty acid moiety.

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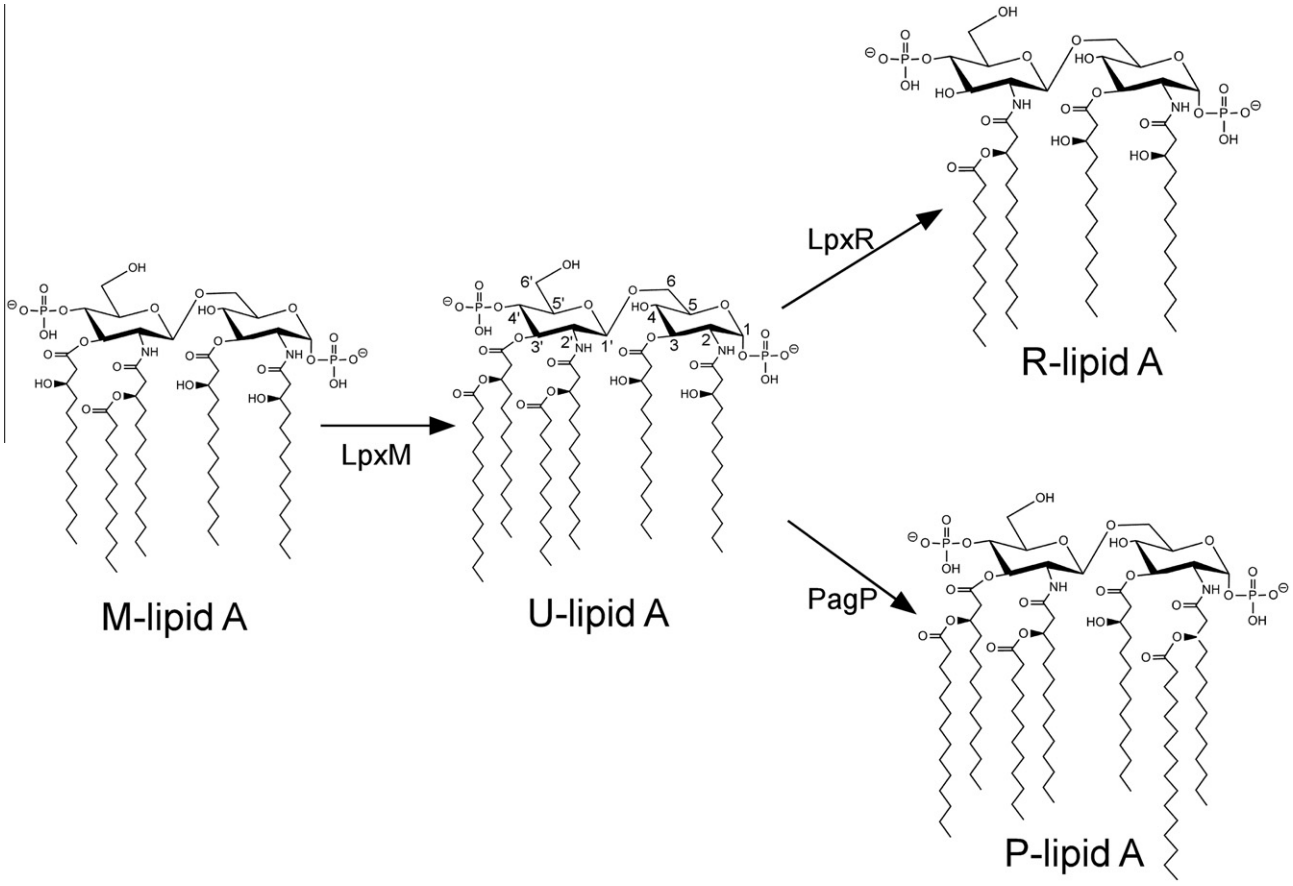

Fig. 1. Structures of the modified lipid A species used in this study. Structures of modified lipid A and the lipid A precursor are shown with the enzymes involved in their synthesis. LpxM catalyzes myristoylation of 3-hydroxymyristate attached at position 3′ of M-lipid A in the bacterial inner membrane. U-lipid A coupled to core oligosaccharide (not shown) at 6′ position was assembled with O-antigen to form the mature LPS molecule in the bacterial inner membrane, which is then transported to the outer membrane. LpxR removes an acyl chain from 3′ position in the outer membrane. PagP catalyzes palmitoylation of 3-hydroxymyristate at 2 position in the outer membrane.

eties in lipid A can be modified by outer membrane enzymes such as palmitoyltransferase PagP, 3-*O*-deacylase PagL, and 3′-*O*-deacylase LpxR [12–15]. Lipid A palmitoylation by PagP and lipid A 3-*O*-deacylation by PagL reduce the ability of lipid A to induce cellular signaling through the TLR4-MD-2 complex, indicating that these modifications are beneficial for invading bacteria to evade host immune surveillance [16]. Furthermore, LpxR-dependent lipid A 3′-*O*-deacylation enhances intracellular growth of *S.* Typhimurium [17]. To date, the effects of lipid A 3′-*O*-deacylation on the bioactivity of lipid A remains to be studied.

In this study, we purified 3′-*O*-deacylated lipid A, unmodified lipid A, palmitoylated lipid A, and a lipid A biosynthetic precursor lacking myristate moiety from *E. coli* and *E. coli* expressing recombinant modification enzymes. A comparison of the ability of 3′-*O*-deacylated lipid A to stimulate the human TLR4-MD-2 complex with those of other purified lipid A species revealed that 3′-*O*-deacylation significantly reduce the bioactivity of lipid A.

2. Materials and methods

2.1. Reagents

Restriction endonucleases and DNA modifying enzymes were obtained from New England Biolabs and Takara Biotech. Silica Gel 60 thin layer chromatography (TLC) plates and high performance thin layer chromatography (HPTLC) plates were obtained from Merck. Oligonucleotides were prepared commercially by Greiner Japan.

2.2. Plasmids

The LpxR coding region was amplified from genomic DNA prepared from *Salmonella enterica* serovar Typhimurium 14028s [18] by PCR using *Pfu* turbo (Stratagene). The primers used for the PCR were KK200 that contained a NcoI site (5′-tcaccatggtgaacaaatacagctattgcg-3′) and KK201 that contained an XbaI site (5′-gactctagatcagaagaagaaggtgatgtc-3′). To generate the expression construct pBAD24-LpxR, the reaction product was cloned into the NcoI/XbaI site of pBAD24 under control of the arabinose pBAD promoter [19]. The expression construct was verified by sequencing.

2.3. Purification and quantification of lipid A

Fig. 1 shows the chemical structures of the lipid A species used in this study. Preparations of unmodified lipid A (U-lipid A) and palmitoylated lipid A (P-lipid A) were performed as described previously with some modifications [16]. In brief, lipid A was prepared from the full-growth culture of *E. coli* XL1-Blue, dissolved in chloroform/methanol (4:1, v/v), and spotted on Silica Gel 60 TLC plates. The plate was developed in chloroform/pyridine/88% formic acid/methanol/water (60:35:10:5:2, v/v) and the band containing lipid A was transiently visualized by spraying water. Extraction of lipid

A from the silica was performed as described previously [20]. For the preparation of P-lipid A, *E. coli* XL1-Blue transformed with pBAD24-PagP was cultivated until an OD_{600} ~0.5 was reached, and then the expression of recombinant *Salmonella* PagP was induced by the addition of L-(+)-arabinose (2 mg/ml). After cultivation for 4 h in the presence of arabinose, the bacteria were collected and lipid A was developed as described above using two sequential rounds of development on Silica Gel 60 TLC plates.

For the preparation of 3′-*O*-deacylated lipid A (R-lipid A), *E. coli* XL1-Blue transformed with pBAD24-LpxR was cultivated to OD_{600} ~0.5 and the expression of recombinant *Salmonella* LpxR was induced by the addition of L-(+)-arabinose (2 mg/ml). After cultivation for 4 h in the presence of arabinose, R-lipid A was prepared from the bacteria that was spotted on Silica Gel 60 TLC plate, and the plate was developed in chloroform/pyridine/88% formic acid/water (50:50:16:5, v/v). After spraying water, lipid A was extracted from the bands and R-lipid A was identified by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) (see below). Extracted R-lipid A was further purified by sequential rounds of development on Silica Gel 60 TLC plates as described above.

For the preparation of the lipid A precursor lacking myristoyl fatty acid moiety (M-lipid A), lipid A was prepared from a full-growth culture of *E. coli* strain JW1844 [21], which expressed a null-mutation in *lpxM*. Strain JW1844 was obtained from National BioResource Project (NIG, Japan). M-lipid A was prepared from the collected bacteria by TLC followed by development in chloroform/pyridine/88% formic acid/methanol/water (60:35:10:5:2, v/v). After visualization by spraying water, lipid A was extracted from the bands and M-lipid A was identified by MALDI-TOF MS.

Quantification of lipid A was performed by measuring the amount of phosphate as described previously [16].

2.4. MALDI-TOF MS

Samples were dissolved in 20 mg/ml of 5-chloro-2-mercapto-benzothhiazole matrixes in chloroform/methanol (1:1, v/v). Spectra were obtained in the negative reflection mode using a Voyager-DE STR mass spectrometer (Applied Biosystems). Each spectrum was the average of 200 shots.

2.5. Luciferase assay

Ba/hTLR4/hMD2 cells, stable Ba/F3 transformants expressing human TLR4, human MD-2 and the luciferase reporter construct p55IGkLuc, were maintained as described previously [22]. Cells (100 μl) were cultured in a 96-well plate and lysed by the addition of 25 μl of 5× cell lysis reagent (Promega). Luciferase activity was measured using 10 μl of cell lysate and 50 μl of luciferase assay substrate (Promega). The luminescence was quantified with a Multi-mode microplate reader (Molecular Devices).

3. Results

3.1. Purification of R-lipid A, P-lipid A, M-lipid A, and U-lipid A

In order to examine the effect of 3′-*O*-deacylation lipid A by LpxR on the bioactivity of LPS, we purified R-lipid A and U-lipid A (Fig. 1). We also purified P-lipid A and M-lipid A (Fig. 1), which are known to be inactive lipid A species against human cells.

R-lipid A was generated in *E. coli* transformed by pBAD24-LpxR for the expression of *S.* Typhimurium LpxR. The negative-ion spectrum of lipid A prepared from *E. coli* transformed with pBAD24-LpxR demonstrated major peaks at *m/z* 1361.4 and 1797.7, and their structures were interpreted as R-lipid A and U-lipid A, respectively (Fig. 2A). U-lipid A and M-lipid A were prepared from *E. coli* and *E. coli lpxM*-null mutant, respectively. The negative-ion spectrum of lipid A prepared from the *E. coli lpxM*-null mutant demonstrated a major peak at *m/z* 1587.2 that was interpreted as M-lipid A (Fig. 2B). In addition, the peak at *m/z* 1825.3 that was interpreted as palmitoylated M-lipid A, was detected in lipid A prepared from the *E. coli lpxM*-null mutant (Fig. 2B). This observation suggested that *E. coli* PagP, which is usually latent [23], palmitoylates M-lipid A in the *lpxM*-null mutant. P-lipid A was generated in *E. coli* transformed with the expression construct pBAD24-PagP as described previously [16].

The negative-ion spectrum of the purified lipid A preparations demonstrated major peaks at *m/z* 1798.0, 1361.6, 2036.0, and 1587.4, and their structures were consistent with those of U-lipid A, R-lipid A, P-lipid A, and M-lipid A, respectively (Fig. 3A). To further ensure that individual lipid A species were isolated, 3 μg of the purified lipid A preparations were developed on TLC and the plates were visualized. As shown in Fig. 3B, each purified lipid A preparation showed a single band with similar intensities. These results, taken together, indicated that each lipid A species was highly purified.

3.2. R-lipid A is less active than U-lipid A, but more active than P-lipid A and M-lipid A, in the induction of cellular signaling via TLR4

To investigate the effect of 3′-*O*-deacylation of lipid A, we examined whether the modification of lipid A changed its ability to induce NF-κB activation through TLR4. The Ba/hTLR4/hMD2 cell line, which is the mouse pro-B cell line Ba/F3 that has ectopic expression of human TLR4 and human MD-2 and is responsive to LPS, was used for the analysis [24]. The cells were stimulated with the purified lipid A species and NF-κB-dependent reporter activation was measured. In Ba/hTLR4/hMD2 cells, purified U-lipid A induced NF-κB activation in a dose-dependent manner and it was

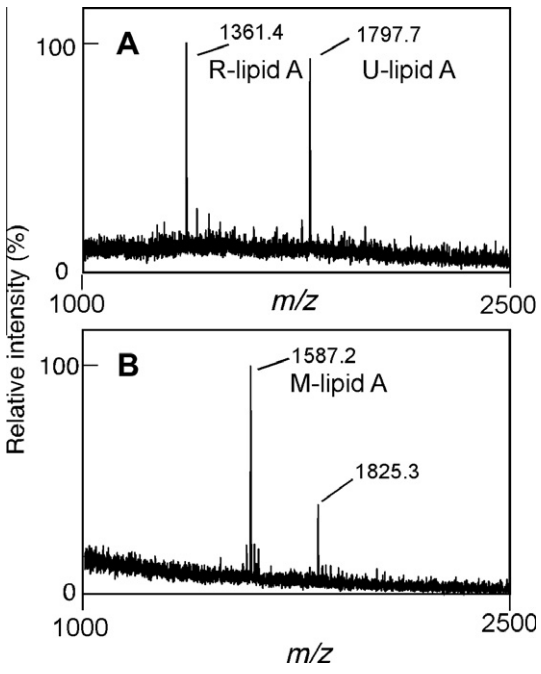

Fig. 2. MALDI-TOF MS analysis of lipid A species in *E. coli* transformed with pBAD24-LpxR and in *E. coli lpxM*-null mutant. Lipid A was prepared from *E. coli* transformed with the expression construct pBAD24-LpxR (A) and *E. coli lpxM*-null mutant (B) by a Tri-reagent-based procedure as described previously [27,28]. MALDI-TOF MS analysis of lipid A preparations are shown and *m/z* values are indicated with interpreted lipid A species.

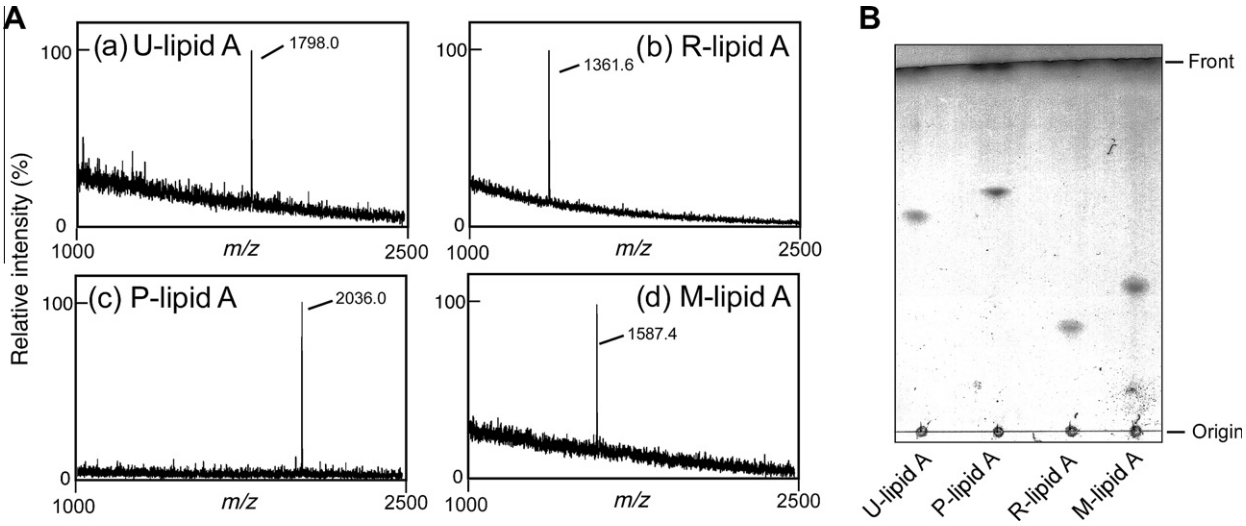

Fig. 3. Purification of lipid A species. (A) a. U-lipid A, b. R-lipid A, c. P-lipid A, and d. M-lipid A purified from *E. coli*, *E. coli* transformed with pBAD24-LpxR, *E. coli* transformed with pBAD24-PagP, and *lpxM*-null *E. coli* mutant, respectively, were suspended in water and incubated with an ion-exchanger, Amberlight® IR120B (Organo Corp.) for 1 h at room temperature. Then, the lipid A preparations were dried and analyzed by MALDI-TOF MS. (B) U-lipid A, P-lipid A, R-lipid A and M-lipid A (3 μg) were spotted onto a HPTLC plate. The plate was developed in chloroform/pyridine/88% formic acid/water (50:50:16:5, v/v). The spots were visualized by heating the plate at 130 °C for approximately 60 min after spraying with a mixture of methanol/sulfuric acid/acetic acid/*p*-anisaldehyde (90:5:5:0.5, v/v).

the most active among the purified lipid A species (Fig. 4). In addition, the Ba/hTLR4/hMD2 cells showed an approximately 4-fold increase of reporter activation relative to basal activity in response to 0.1 ng/ml U-lipid A and 3.3 ng/ml R-lipid A stimulation (Fig. 4), indicating that R-lipid A is approximately 30-fold less active than U-lipid A (Fig. 4). In contrast, approximately 4-fold increased induction of reporter activation was observed in response to 100 ng/ml P-lipid A and 330 ng/ml M-lipid A (Fig. 4), indicating that R-lipid A is approximately 30- to 100-fold more active than P-lipid A and M-lipid A. These results were consistent with previous observations that P-lipid A was less active than U-lipid A in the stimulation of human TLR4-MD-2 complex or human macrophage-like cell line [16,25]. Moreover, relative to LPS prepared from wild-type *S.* Typhimurium and *E. coli*, LPS prepared from the *lpxM*-null mutant showed dramatically reduced activity for the induction of cytokines from human macrophage-like cell lines and stimulation of human endothelial cells [6,7]. These results, taken together, indicated that 3′-*O*-deacylation reduced the ability of lipid A to stimulate human TLR4-MD-2 complex. Despite the reduced activity of R-lipid A, it had more activity than the inactive lipid A species, P-lipid A and M-lipid A.

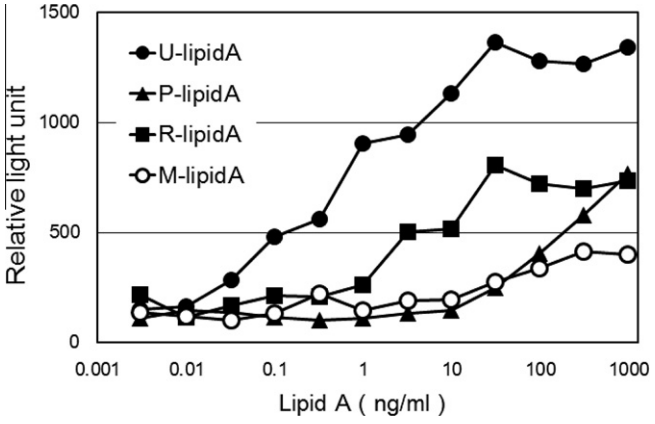

Fig. 4. R-lipid A is less active than U-lipid A, but more active than P-lipid A and R-lipid A, for the induction NF-κB activation through TLR4. Ba/hTLR4/hMD2 cells were cultivated for 4 h in culture medium containing the indicated amount of U-lipid A (closed circle), P-lipid A (closed triangle), R-lipid A (closed square), or M-lipid A (open circle), and luciferase activity was measured. Data represent the average of triplicate assays and the results shown are representative of three independent experiments.

4. Discussion

In this study, we generated and/or purified R-lipid A, P-lipid A, M-lipid A, and U-lipid A, in order to compare their ability to stimulate the human TLR4-MD-2 complex. Our results indicated that lipid A 3′-*O*-deacylation by LpxR significantly reduces its ability to stimulate the human TLR4-MD-2 complex, thus indicating that lipid A 3′-*O*-deacylation by LpxR is beneficial for evasion of the host immune surveillance by invading *Salmonella*. On the other hand, the effect of lipid A 3′-*O*-deacylation by LpxR reduced the bioactivity of lipid A less than lipid A palmitoylation by PagP. A previous study demonstrated that lipid A 3′-*O*-deacylation by LpxR occurred in tandem with lipid A palmitoylation in *S.* Typhimurium [17]. It is possible that lipid A modifications, such as palmitoylation by PagP and 3′-*O*-deacylation by LpxR, cooperatively affect the bioactivity of lipid A. The combination of lipid A modifications likely promote bacterial adaptation to host tissues, and the effect of the modifications on bacterial pathogenesis remains to be determined.

Interestingly, M-lipid A that has 3-hydroxymyristate moiety at 3′ position of R-lipid A is significantly less active than R-lipid A (Fig. 1). Thus, suggesting that the reduction of the number of fatty acid moieties on lipid A does not simply reduce the bioactivity of lipid A. The 3-hydroxymyristate moiety may be important to reduce the ability of M-lipid A to stimulate the human TLR4-MD-2 complex compared to R-lipid A. The structural basis for the sensing of lipid A and its derivatives by TLR4-MD-2 complex has been solved by X-ray analysis of the crystal structures and the 3-hydroxymyristate moiety at the 3′ position of lipid A has been shown to interact with MD-2 [3,26]. This interaction may be responsible for the reduced ability of M-lipid A to stimulate human TLR4-MD-2 complex relative to R-lipid A. In addition, it is likely involved in the previously reported antagonistic activity of LPS prepared from *lpxM*-null mutant [7]. The TLR4-MD-2 complex has been shown to be a druggable target for the treatment of septic shock and as vaccine adjuvants. Therefore, our observations

concerning the bioactivity of R-lipid A and M-lipid A will be valuable for the development of drugs that target TLR4-MD-2 complex.

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