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Preclinical report

A signaling pathway by a new synthetic lipid A analog, ONO-4007, in RAW264.7 cells

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ONO-4007 is a new synthetic lipid A derivative with low endotoxic activities. We have reported that ONO-4007 could be a new biological response modifier for the treatment of tumor necrosis factor (TNF)-α-sensitive tumors. In this study, we confirmed that ONO-4007 activated a murine macrophage cell line, RAW264.7, and that the activated RAW264.7 cells produced TNF-α *in vitro*. RAW264.7 cells stimulated for less than 15 min with ONO-4007 (40 μg/ml) did not produce TNF-α (less than 4 U/ml). However, 24 h stimulation with ONO-4007 induced TNF-α production (more than 256 U/ml) in RAW264.7 cells. Although P38 in mitogen-activated protein kinase of the RAW264.7 cells was not tyrosine phosphorylated by ONO-4007 stimulation, ERK1 of the RAW264.7 cells was tyrosine phosphorylated for 5–15 min by ONO-4007 stimulation. Tyrosine phosphorylation of ERK1 decreased gradually from 15 min after stimulation and almost disappeared 60 min after stimulation. These findings indicate that ONO-4007 stimulates RAW264.7 cells immediately and induces tyrosine phosphorylation of ERK1 in the RAW264.7 cells for 5–15 min. These data suggest that the signal transduction pathway of ONO-4007 may be similar to that of lipopolysaccharide. [© 2002 Lippincott Williams & Wilkins.]

Key words: ERK1, lipopolysaccharide, ONO-4007, RAW264.7 cell.

Introduction

Lipopolysaccharide (LPS) in a Gram-negative bacterial envelope is a potent stimulator for many early events in macrophage activation.[1,2] Many investigators showed that LPS also has potent antitumor activities.[2,3] However, since LPS causes severe side effects, it is not yet used clinically. Lipid A, a hydrophobic component of bacterial LPS, is known to be a biologically active site of LPS. A number of synthetic lipid A derivatives have been developed to be less harmful to hosts, but they have not reached satisfactory levels either in terms of potent immunopharmacological activities or low toxicity.[4–6] A novel lipid A derivative, ONO-4007, has toxicity as low as less than 1/1000 that of natural *Escherichia coli* LPS.[7] However, the mechanism of activation of macrophages by ONO-4007 has not fully been elucidated. We used a murine macrophage cell line, RAW 264.7, to analyze the activation mechanism of ONO-4007. Here we describe a signaling pathway by ONO-4007 stimulation in RAW 264.7 cells.

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Materials and methods

Cells and cell culture

The murine macrophage cell line RAW 264.7 and the murine fibroblast cell line L929 cells were cultured (37°C, 5% CO_2) in RPMI 1640 culture medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum, 2 mM L-glutamine and antibiotics.

Reagents

ONO-4007 (sodium-2-deoxy-2-[3s-(9-phenylnonayloxy)tetradecanoyl]-amino-3-*O*-(9-phenylnona-nonyl)-D-glucopyranose4-sulfate) was kindly provided by ONO Pharmaceutical (Osaka, Japan). Figure 1 shows its chemical structure. ONO-4007 was dissolved in

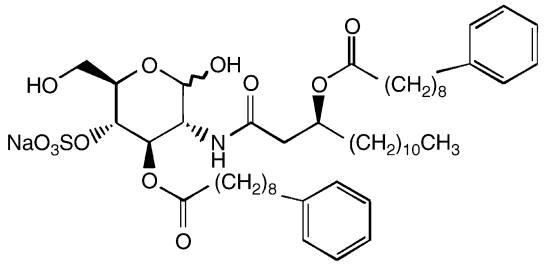

Figure 1. Chemical structure of ONO-4007.

dimethyl sulfoxide (DMSO) at 50 mg/ml, diluted with RPMI 1640 medium supplemented with 10% FBS. Recombinant human tumor necrosis factor (TNF)-α was purchased from Boehringer Mannheim (Mannheim, Germany; 1371843). Anti-ERK1 polyclonal antibody and anti-p38 monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Bioassay for TNF-α

The TNF-α assay employed in this experiment was the 1-day assay using L-929, a TNF-α-sensitive murine fibroblast cell line, which was carried out in the presence of 8.0 μg/ml actinomycin D.[8] Test samples were obtained from the culture supernatant of RAW 264.7 cells at 15 min and 24 h after the administration of ONO-4007 (40 μg/ml).

Preparation of cell lysates

RAW264.7 cells (2×10^7) were incubated in the presence of ONO-4007 (40 μg/ml) for 1, 5, 15, 30 and 60 min. They were then harvested and centrifuged at 10 000 r.p.m. for 10 s. The pellet was washed with 1 ml of ice-cold TBS, pH 7.4. After centrifugation (10 000 r.p.m., 10 s) the cells were lysed in 1200 μl of Nonidet P-40 lysis buffer (165 mM NaCl, 50 mM Tris–HCl, pH 7.5, 1% Nonidet P-40, 10 mM EDTA, 10 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin) at 4°C for 1 h. After centrifugation (15 000 r.p.m., 20 min, 4°C) the supernatants were stored at −80°C until use.

SDS–PAGE and immunoblot

SDS–PAGE was carried out using separating gels of 15.0%T and 2.6%C according to the method of Laemmli.[9] The protein in the gel was transferred onto a PVDF membrane (Millipore, Bedford, MA).[10] After blocking with TBS containing 5% skim milk, the membrane was allowed to react with the first antibody 4G10 (anti-phosphotyrosine antibody) for 1 h. The membrane was washed with TBS containing 0.05% Tween 20 and allowed to react with the second antibody (peroxidase-conjugated goat anti-mouse antibody) for 1 h. The membrane was washed again and immunoreacted substances were visualized by enhanced chemiluminescence using a detection kit (Amersham Pharmacia Biotech, Little Chalfont, UK).

Antibody pull-down assay

Lysates of RAW264.7 (2×10^7), treated with ONO-4007 (40 μg/ml) for 15 min, were prepared and immunoprecipitated as previously described.[11] Protein A–Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden) (50 μl per precipitation) precoated with 5 μg anti-p38 mAb or anti-ERK1 antibody were used for the assay. The cell lysates (2×10^7 cells) were incubated with the antibody-coated Protein A–Sepharose beads at 4°C for 1 h and centrifuged. A part of the supernatant was used as a sample. The rest of lysates were then incubated with the new antibody-coated Protein A–Sepharose beads again, at 4°C for 1 h, and centrifuged. A part of the supernatant was likewise used as a sample and these procedures were repeated. The samples were separated by SDS–PAGE,[10] transferred to PVDF membranes (Millipore) and immunoblotted as described previously.[11] Then 30 μl of SDS sample buffer was added to the used beads for the pull-down assay and treated at 95°C for 5 min, and centrifuged at 10 000 r.p.m. for 15 s at 4°C. The supernatants were used for SDS–PAGE as samples after being heated at 95°C for 5 min and mixed with 5 × SDS sample buffer.[10]

Results

TNF-α production of RAW264.7. cells by ONO-4007 stimulation

TNF-α production was measured by bioassay using L929 cells. More than 256 U/ml of TNF-α was

produced in the supernatant of RAW264.7 cells treated with ONO-4007 (40 μg/ml) for 24 h. However, RAW264.7 cells without ONO-4007 treatment produced less than 4 U/ml TNF-α. TNF-α activity in the supernatant of RAW264.7 cells treated with ONO-4007 for 15 min was not detected (below 4 U/ml) (data not shown).

Immunoblot analysis

Figure 2 shows that ONO-4007 treatment (40 μg/ml) induced tyrosine phosphorylation of a 42-kDa protein in RAW264.7. cells. This tyrosine phosphorylation began 5 min after the treatment and came to an end 30 min after the treatment.

Antibody pull-down assay

To identify this 42-kDa tyrosine-phosphorylated protein, we performed an antibody pull-down assay. Since some groups reported that LPS stimulated the mitogen-activated protein (MAP) kinase pathway, the anti-p38 antibody and anti-ERK 1 antibody were used in this assay. Figure 3 shows that the expression of 42-kDa tyrosine-phosphorylated protein from the lysate did not decrease after immunoprecipitation with anti-p38 antibody-coated Protein A–Sepharose beads. Figure 4 shows that the expression of the 42-kDa tyrosine-phosphorylated protein in the lysate faded after immunoprecipitation with anti-ERK 1 antibody-coated Protein A–Sepharose beads. Figure 5 shows that the 42-kDa tyrosine-phosphorylated protein was immunoprecipitated with the used anti-ERK 1 antibody-coated Protein A–Sepharose beads in this pull-down assay.

Discussion

Bacterial compounds such as LPS, lipoproteins of different sources and other bacterial cell wall components or synthetic lipopeptides are known to stimulate macrophages[12–14] which are linked to the regulation and expression of cytokine genes as well as to other important cellular functions. Whereas appropriate amounts of inflammatory cytokines such as interleukin (IL) and TNF-α induced by LPS activate immune systems, excessive inflammatory cytokines cause grave conditions (fever, shock and blood coagulation). We carried out the experiment with a novel lipid A derivative, ONO-4007, which has a low toxicity less than 1/1000 that of natural *E. coli* LPS.[7] The focus of this study was to elucidate the signaling mechanism of ONO-4007-induced activation of macrophages. There are many *in vitro* and *in vivo* reports about ONO-4007;[15–18] however, this report is

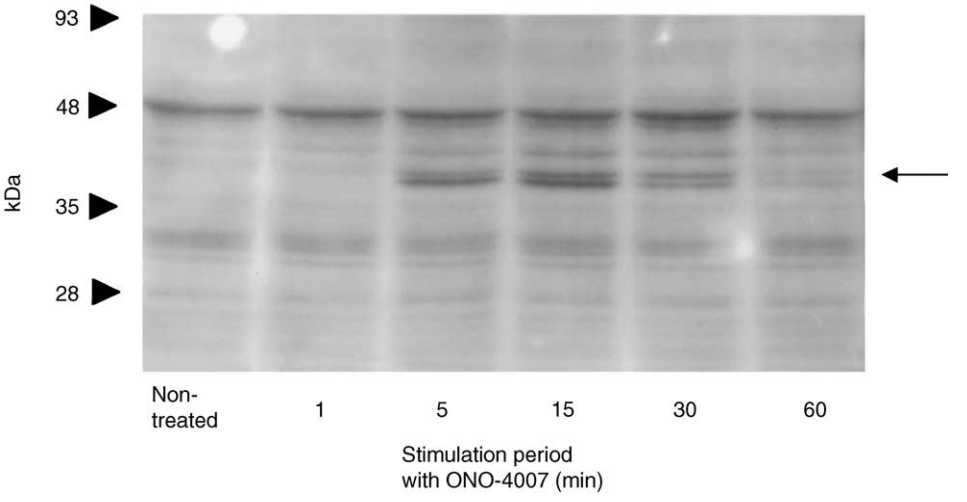

Figure 2. Western blot of the lysate of ONO-4007-stimulated RAW264.7 cells reactive with 4G10. RAW264.7 cells were incubated with ONO-4007 (40 μg/ml) for 1, 5, 15, 30 and 60 min. They were harvested and centrifuged, and the pellet was washed. After centrifugation, the cells were lysed in Nonidet P-40 lysis buffer at 4°C for 1 h. After centrifugation the supernatants were used for SDS–PAGE. The proteins in the gel were transferred to a PVDF membrane. After blocking, the membrane was allowed to react with 4G10. The membrane was washed and set to react with the second antibody. The membrane were washed again and immunoreactive substances visualized by enhanced chemiluminescence

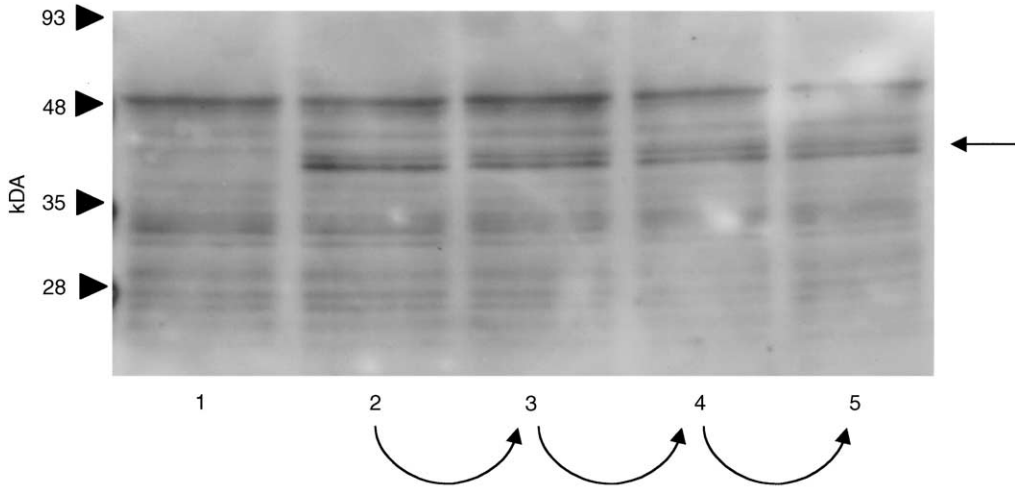

Figure 3. Anti-p38 antibody pull-down assay for the lysate of ONO-4007-stimulated RAW264.7 cells. Lysate of RAW264.7 (2×10^7) treated with ONO-4007 (40 μg/ml) for 15 min was prepared and immunoprecipitated. Protein A–Sepharose beads precoated with 5 μg anti-p38 antibody were used for the first assay. The cell lysates were incubated with the antibody-coated Protein A–Sepharose beads and centrifuged. A part of the supernatant was used as a sample. The rest of the lysates were then incubated with new antibody-coated Protein A–Sepharose beads again and centrifuged. A part of the supernatant was used as a sample. These procedures were repeated. The samples were separated by SDS–PAGE, and transferred onto PVDF membranes and immunoblotted. Lane 1: cell lysate from untreated RAW264.7 cells. Lane 2: cell lysate from ONO-4007-treated RAW264.7 cells. Lane 3: the rest of the sample shown in lane 2, after the immunoprecipitation with anti-p38 antibody-coated beads. Lane 4: the rest of the sample shown in lane 3, after the immunoprecipitation with anti-p38 antibody-coated beads. Lane 5: the rest of the sample shown in lane 4, after the immunoprecipitation with anti-p38 antibody-coated beads.

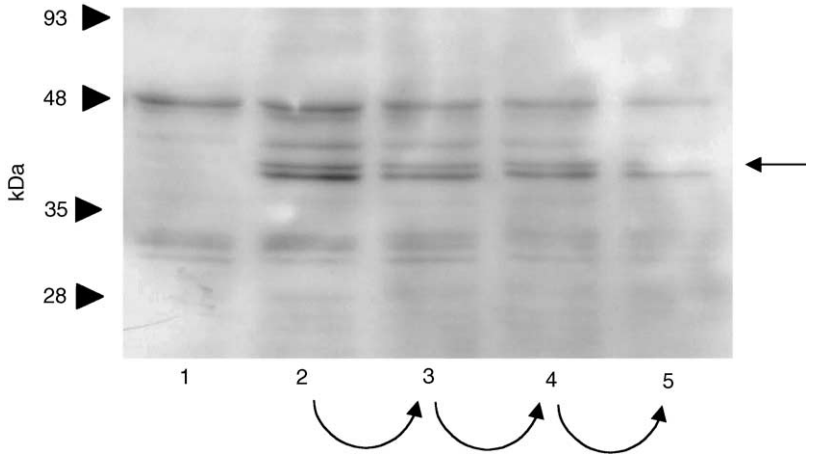

Figure 4. Anti-ERK1 antibody pull-down assay for the lysate of ONO-4007-stimulated RAW264.7 cells. Lysate of RAW264.7 (2×10^7) treated with ONO-4007 (40 μg/ml) for 15 min was prepared and immunoprecipitated. Protein A–Sepharose beads precoated with 5 μg anti-ERK1 antibody were used for the first assay. The cell lysates were incubated with the antibody-coated Protein A–Sepharose beads and centrifuged. A part of the supernatant was used as a sample. The rest of lysates were then incubated with new antibody-coated Protein A–Sepharose beads again and centrifuged. A part of the supernatant was used as a sample. These procedures were repeated. The samples were separated by SDS–PAGE, and transferred onto PVDF membranes and immunoblotted. Lane 1: cell lysate from untreated RAW264.7 cells. Lane 2: cell lysate from ONO-4007-treated RAW264.7 cells. Lane 3: the rest of the sample shown in lane 2, after the immunoprecipitation with anti-ERK1 antibody-coated beads. Lane 4: the rest of the sample shown in lane 3, after the immunoprecipitation with anti-ERK1 antibody-coated beads. Lane 5: the rest of the sample shown in lane 4, after the immunoprecipitation with anti-ERK1 antibody-coated beads.

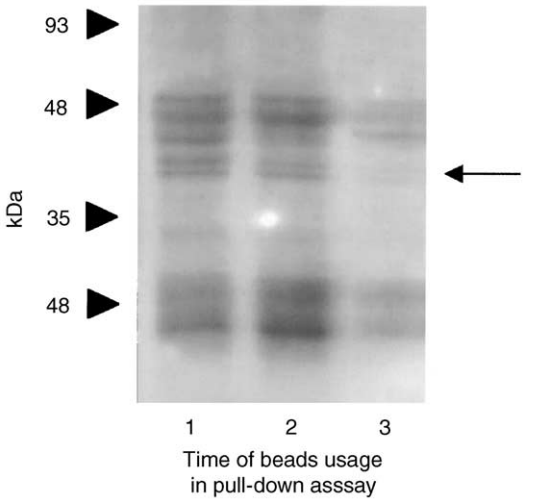

Figure 5. Western blot of the immunoprecipitates from the beads coated with anti-ERK1 antibody. The beads used in the anti-ERK1 antibody pull-down assay were added to the sample buffer, which was then heated at 95°C for 5 min and centrifuged. The supernatants were separated by SDS–PAGE and the proteins in the gel were transferred to a PVDF membrane. After blocking, the membrane was allowed to react with 4G10. The membrane was washed and set to react with the second antibody. They were washed again and immunoreactive substances were visualized by enhanced chemiluminescence. Lane 1: immunoprecipitate from the first immunoprecipitation with anti-ERK1 antibody-coated beads. Lane 2: immunoprecipitate from the second immunoprecipitation with anti-ERK1 antibody-coated beads. Lane 3: immunoprecipitate from the third immunoprecipitation with anti-ERK1 antibody-coated beads.

the first to demonstrate a part of the signaling mechanism. Since we had never used RAW264.7 cells for the study of ONO-4007, we examined if ONO-4007 activated these cells by measuring the TNF activity in their supernatant 24 h after the ONO-4007 stimulation. The TNF bioassay using L929 cells showed that ONO-4007 activated RAW264.7 cells and the activated RAW264.7 cells produced a large amount of TNF-α. Then the ONO-4007 treatment induced tyrosine phosphorylation of a 42-kDa protein. We observed the kinetics of ONO-4007-induced tyrosine phosphorylation of this 42-kDa protein. It was clear that ONO-4007 stimulation induced tyrosine phosphorylation in RAW 264.7 cells until 30 min after stimulation and then this phosphorylation decreased. One minute after ONO-4007 stimulation this tyrosine phosphorylation was not detected (Figure 2). Many reports show that LPS activates p38, JNK and ERK in terms of their signal transduction.[19,20] An anti-p38 antibody pull-down assay was performed which revealed no change in the tyrosine-phosphorylated bands (Figure 3). Next we performed an anti-ERK1 antibody pull-down assay. As immunoprecipitation with anti-ERK1-coated beads was repeated, the tyrosine-phosphorylated bands became fainter (Figure 4). It was evident that the 42-kDa tyrosine-phosphorylated protein band was MAP kinase and this MAP kinase was ERK1. However, only ERK1 in the MAP kinase was activated in signal transduction by ONO-4007. Schumann *et al.* reported that MAP kinase was tyrosine phosphorylated from 5 to 15 min after stimulation with LPS.[19,21] In our study, ERK1 in RAW264.7 cells was tyrosine phosphorylated likewise from 5 min after stimulation with ONO-4007 (Figure 2). We then measured TNF-α activity in the culture supernatant of RAW264.7 cells 15 min after ONO-4007 administration (40 μg/ml). RAW264.7 cells stimulated with ONO-4007 for 15 min did not produce TNF-α, i.e. it needed more than 15 min for RAW264.7 cells to produce TNF-α by ONO-4007 stimulation. Although ONO-4007 stimulation induced tyrosine phosphorylation of MAP kinase in 15 min, it was not dependent on the autocrine system of TNF-α production by RAW264.7 cells stimulated by ONO-4007. These data also indicate a high possibility that ONO-4007 and LPS may have similar signaling mechanisms for macrophage activation. LPS activates NF-κB and MAP kinase through Toll-like receptor (TLR) 4 receptor, resulting in induced acquired immunity.[22–24] It has been reported that ONO-4007 can induce a specific immune response; however, the details are not known.[18] They indicate that ONO-4007 and LPS have similar signaling mechanisms. It is thought that the signaling cascades through TLR4 receptor can lead to activation of NF-κB, and MAP kinase, p38, JNK and ERK1.[25–27] Among the signal cascades through TLR4, the one associated with myeloid differentiation factor (MyD88), IL-1 receptor-associated kinase (IRAK) and TNF receptor-associated factor 6 (TRAF6) is well known. Other cascades through the TLR4 receptor[28,29] are not yet well known. Human TLR4 receptor, the mammalian homolog of Toll, was first identified by Janeway's group.[30] To date, more than 10 members have been reported to belong to the TLR family in mammals. Apart from TLR4, it might be possible that some other TLRs may associate with ONO-4007. It will be useful to elucidate the signal transduction mechanisms of ONO-4007 for practical use of the drug and development of other drugs for the immunological control system, and further to clarify the signal transduction mechanisms of TLR receptors.

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