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Analgesic effects of chemically synthesized NOD1 and NOD2 agonists in mice

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Intracellular nucleotide-binding oligomerization domain (NOD)-like receptors, NOD1 and NOD2, recognize the diaminopimelic acid (DAP)-containing peptide moiety and muramyldipeptide (MDP) moiety of bacterial peptidoglycan, respectively. Muramyldipeptide has been reported to exert analgesic activity to decrease the frequency of acetic acid-induced writhing movements in mice. In this study, we demonstrated the analgesic activities of NOD1 as well as NOD2 agonists. Intravenous injection of NOD2-agonistic MDP, 6-*O*-stearoyl-MDP (L18-MDP), and MDP-Lys (L18) exhibited analgesic activity at 10, 50, and 2.0 μg/head, respectively, in BALB/c mice. NOD1-Agonistic FK156 (D-lactyl-L-Ala-D-Glu-*meso*-DAP-L-Gly) and FK565 (heptanoyl-D-Glu-*meso*-DAP-D-Ala) were also analgesic at 50 μg/head and 1.0 μg/head, respectively. The analgesic effect of FK565 appeared from 30 min, reached maximum activity at 8 h, and continued until 24 h. The FK565 exhibited activity by various administration routes; intravenous, intraperitoneal, intramuscular, sublingual (1.0 μg/head each), subcutaneous, intragastric (oral), intragingival (10 μg/head each) and intracerebroventricular (0.01 μg/head). The analgesic activity of FK565 was observed even in tumor necrosis factor (TNF)-α knockout, interleukin (IL)-1α/β double knockout, and their triple knockout mice. Naloxane, a non-selective antagonist for the opioid receptor, completely inhibited the analgesic effect of FK565. These findings suggest that NOD1 and NOD2 activation induces an analgesic effect via opioid receptors in a TNF-α and IL-1α/β-independent manner.

Keywords: NOD1, NOD2, FK565, MDP, analgesic effect, opioid

Introduction

Bacterial peptidoglycans (PGNs) exhibit various immunobiological activities, including adjuvant activity, to induce cell-mediated immunity (delayed-type hypersensitivity) against test protein antigens, which was originally recognized in relation to Freund's complete adjuvant (FCA).[1,2] In the mid-1970s, the minimum essential structure of PGN for the immunoadjuvant activity of FCA was chemically synthesized and designated muramyldipeptide (MDP; *N*-acetylmuramyl-L-alanyl-D-isoglutamine).[3,4] As expected, MDP exhibited most bioactivities of PGN.[5] In 2003, nucleotide-binding oligomerization domain (NOD)2, a protein associated with susceptibility to Crohn's disease, was demonstrated to be an intracellular receptor for MDP.[6,7] Thereafter, a *meso*-diaminopimelic acid (*meso*-DAP)-containing peptide moiety of PGN was found to be recognized by another NOD protein, NOD1.[8,9] In an original study, Fleck *et al.*[10] first reported that desmuramylpeptides (DMP) carrying *meso*-DAP adjuvant-actively induced cell-mediated immunity, although they recognized that lysine-type DMP were similarly active to *meso*-DAP-type DMP in this respect.

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Meso-DAP-type PGN is found in most Gram-negative bacteria and in some Gram-positive bacteria, such as mycobacteria, while L-lysine-type PGN is found in most Gram-positive bacteria.[11] Thereafter, French and Japanese investigators chemically synthesized *meso*-DAP-type DMP.[12] In the course of the study, Fujisawa (Astellas) Pharmaceutical Co.[13] generated a desmuramylpeptide, D-lactyl-L-alanyl-γ-D-glutamyl-*meso*-DAP-glycine by chemically mimicking a counterpart purified from fermentation broths of *Streptomyces* strains and designated it FK156. They then synthesized various derivatives of FK156, among which the leading compound was FK565, heptanoyl-γ-D-glutamyl-*meso*-DAP-D-alanine. They also reported that the minimum active entity of desmuramylpeptide was γ-D-glutamyl-*meso*-DAP,[14] which was later reported to be the minimum structure for NOD-agonist and was designated iE-DAP.[8]

In 1987, Ogawa and Kotani[15] reported that MDP and its analogs exhibited analgesic effects that decreased abdominal writhing movements induced by intraperitoneal injection of acetic acid. Horák and Mašek[16] also reported that MDP and its analog, adamantylamide dipeptide, exhibited analgesic effects. This unique activity of MDP has not been further studied to date. In the present study, we first examined whether various NOD1 and NOD2 agonists other than MDP also exhibited analgesic activities in an acetic acid test. We found that both NOD1 and NOD2 agonists exhibited analgesic activities; in particular, FK565 was the strongest of the tested agonists and exerted analgesic activities when administered via various routes, including orally.

Materials and Methods

Chemicals

Chemically synthesized MDP was purchased from the Protein Research Foundation Peptide Institute (Osaka, Japan). Synthetic MDP derivatives, N^{α}-(*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl)-N^{ε}-stearoyl-L-lysine [MDP-Lys(L18)] and 6-*O*-stearoyl-MDP (L18-MDP) were supplied by Daiichi Pharmaceutical Co. (Tokyo, Japan). The synthetic desmuramylpeptides, FK156 (D-lactyl-L-alanyl-γ-D-glutamyl-*meso*-diaminopimelyl-glycine) and FK565 (heptanoyl-γ-D-glutamyl-*meso*-diaminopimelyl-D-alanine) were supplied by Astellas Pharmaceutical Co. (Tokyo, Japan): the chemical structures of these compounds are shown in Figure 1. These test materials were dissolved in saline. Naloxone hydrochloride, a non-selective antagonist for opioid receptors, was purchased from MP Biomedicals (Illkirch, France).

Animals

Male and female BALB/c mice, 6–8 weeks old and weighing 18–25 g, were obtained from the animal facility of our university, and female mice were mainly used in experiments. BALB/c interleukin (IL)-1 knockout mice (deficient in both IL-1α and IL-1β), TNF-α knockout mice, and IL-1/TNF-α knockout mice (deficient in IL-1α, IL-1β, and TNF-α) were established from the original IL-1α knockout, IL-1β knockout, and TNF-α knockout mice by back-crossing to BALB/c mice.[17,18] Room temperature, humidity and light cycle were controlled to $23 \pm 1^{\circ}$C, $50 \pm 20\%$, and 12 h (lights on 08:00), respectively. Not more than 10 mice were housed per cage and they were given free access to food and water. The following experimental procedures accorded with the Guidelines for the Care and Use of Laboratory Animals in Tohoku University, and were approved by the Ethical Review Board for Experimental Animals of Tohoku University.

Acetic acid test

Mice received an intravenous injection of test materials and, 1 h later, received an intraperitoneal (i.p.) injection of 1% acetic acid (0.1 ml/10 g of body weight). The number of writhing movements was counted during a 20-min period after the acetic acid injection.

Statistical analysis

Results are expressed as the mean values ± SEM, and examined by paired *t*-test in the case of comparison between two groups and one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey multiple comparison test in the case of comparison among three or more groups. A *P*-value of less than 0.05 was considered significant.

Results

Analgesic effects of various NOD2 and NOD1 agonists

First, we examined whether various NOD1 and NOD2 agonists exhibited analgesic effects to decrease writhing movements induced by intraperitoneal injection with acetic acid (Fig. 2). Muramyldipeptide (MDP) inhibited writhing movements in a dose-dependent manner, showing slight and moderate activity at 0.4 μg/head and 2 μg/head, respectively, although the activities were not statistically significant, and significant activity at 10 μg/head. MDP-Lys(L18) and 6-*O*-L18-MDP were

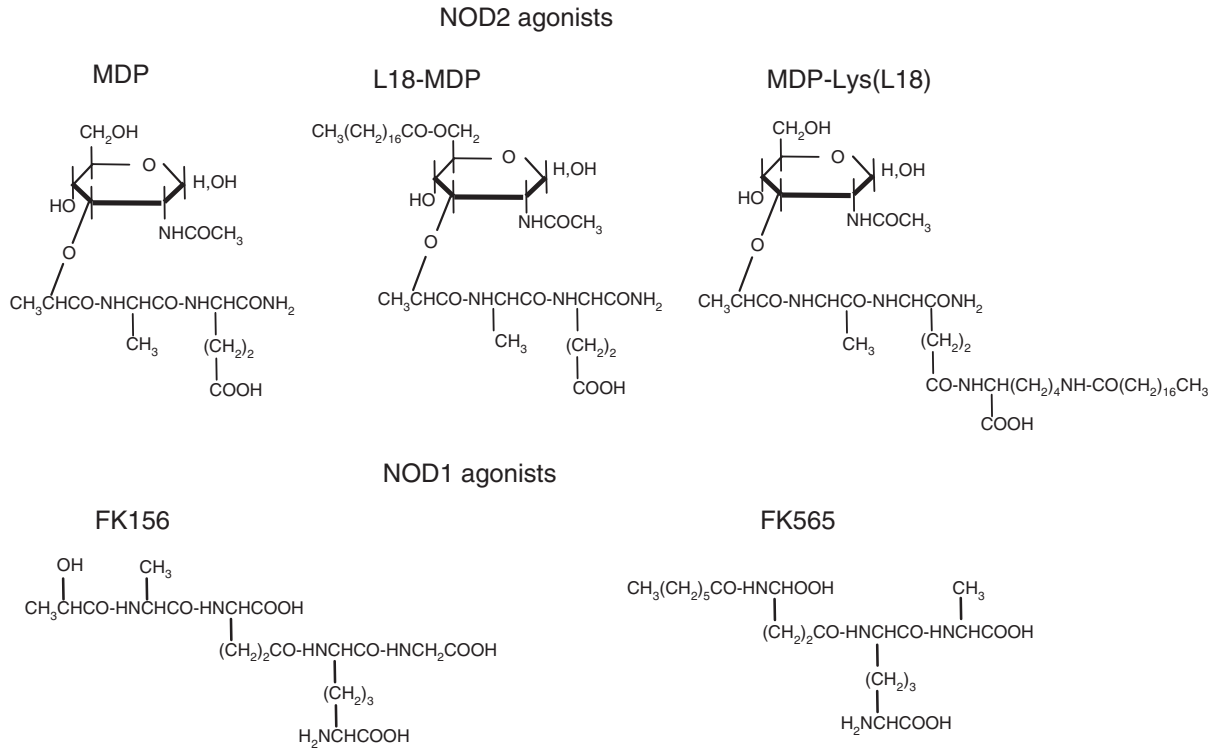

Fig. 1. Chemical structures of synthetic NOD2 and NOD1 agonists. MDP, *N*-acetylmuramyl-L-alanyl-D-isoglutamine; L18-MDP, 6-*O*-stearoyl-MDP; MDP-Lys(L18), N^{α}-(*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl)-N^{ε}-stearoyl-L-lysine; FK156, D-lactyl-L-alanyl-γ-D-glutamyl-*meso*-diaminopimelyl-glycine; FK565, heptanoyl-γ-D-glutamyl-*meso*-diaminopimelyl-D-alanine.

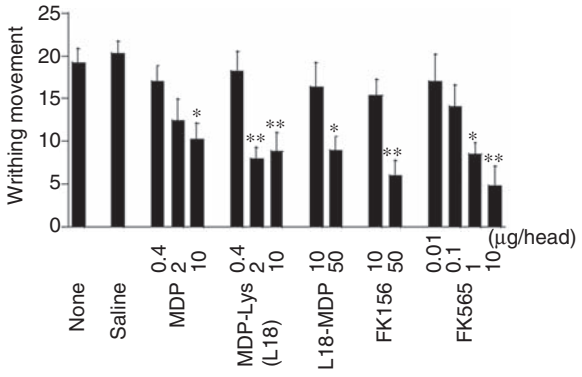

Fig. 2. Analgesic effects of various NOD2 and NOD1 agonists. Groups (6 per group) of female BALB/c mice received an intravenous injection of the indicated dose of test compounds. One hour later, the mice received an intraperitoneal injection of 1% acetic acid (0.1 ml/10 g body weight), and then the number of writhing movements was counted during a 20-min period. $*P<0.05$ and $**P<0.01$ versus control (none).

more and less effective than MDP, respectively, exhibiting significant activity at 2 μg/head and 50 μg/head, respectively. Concerning NOD1 agonists, FK565 was the most powerful compound among the test compounds, exhibiting significant activity even at 1 μg/head (Fig. 2). On the other hand, FK156 was less effective than MDP; 50 μg/head was required to exhibit significant activity. It must be noted here that intravenous injection of saline did not influence writhing movements; no significant difference was observed between the saline-injected group and non-treated group (Fig. 2). It must also be noted that no differences were observed between male and female mice (data not shown).

Time course of analgesic effects of FK565

We then examined the time course of the analgesic effects of FK565 in the acetic acid test. The mice were injected intravenously with FK565 (10 μg/head) and, after various intervals, were subjected to the acetic acid test (Fig. 3). Analgesic effects appeared from 30 min and continued until 24 h. The strongest effect was observed 8 h after FK565 infection. It should be noted that MDP also exhibited a significant analgesic effect at 8 h (Fig. 3).

Analgesic effects of FK565 administered via various routes

Next, we examined whether FK565, the strongest compound, was effective when administered via routes other than intravenous injection. In preliminary experiments, saline administered via routes except the intracerebroventricular (i.c.v.) route did not influence

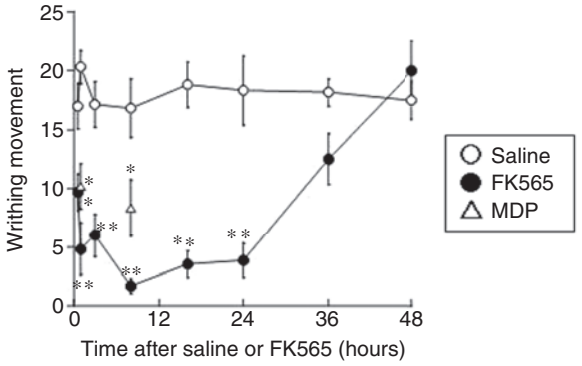

Fig. 3. Time course of the analgesic effect of FK565. Groups (6 per group) of female mice were injected intravenously with FK565 (10 μg/head or saline); at the indicated intervals, they received an intraperitoneal injection of 1% acetic acid (0.1 ml/10 g body weight), and the number of writhing movements was counted during a 20-min period. The results of MDP at 8 h as well as at 1 h are shown. The results at 1 h are from Figure 2. $^{*}P<0.05$ and $^{**}P<0.01$ versus control (saline).

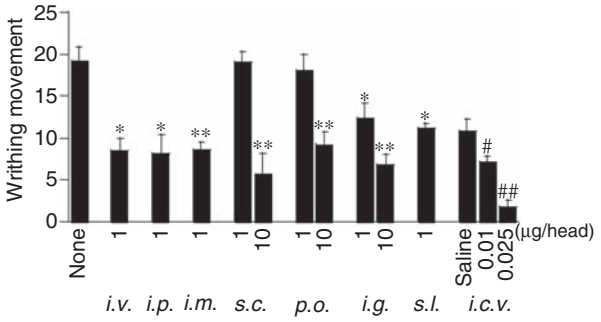

Fig. 4. Analgesic effects of FK565 administered via various routes. Groups (6 per group) of female mice were given FK565 via various routes. One hour later, they received an intraperitoneal injection of 1% acetic acid (0.1 ml/10 g body weight), and then the number of writhing movements was counted during a 20-min period. The results of 'none' and 'i.v.' are from Figure 2. $^{*}P<0.05$ and $^{**}P<0.01$ versus none; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ versus saline.

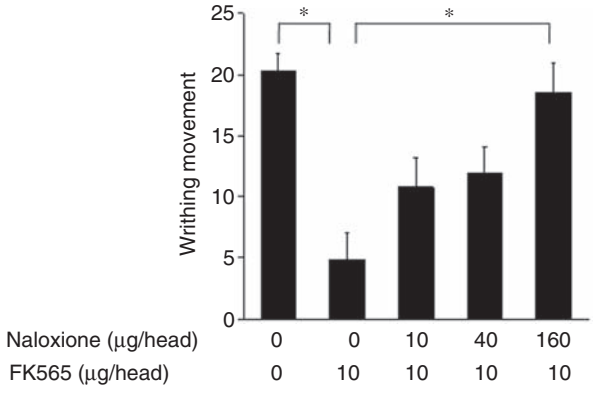

Fig. 5. Reduction of analgesic effects of FK565 by naloxone. Groups (6 per group) of mice were injected subcutaneously with naloxone or saline, and 10 min later were injected intravenously with FK565 (10 μg/head) or saline. One hour later, the mice received an intraperitoneal injection of 1% acetic acid (0.2 ml/head), and the number of writhing movements was counted during a 20-min period. $^{*}P<0.05$ between two groups.

writhing movement (data not shown); therefore, a comparison was generally performed with the non-treated group, as shown in Figure 2. Intraperitoneal (i.p.) and intramuscular (i.m.) injections of FK565 were similarly effective as intravenous injection; significant activity was observed at 1 μg/head (Fig. 4). Subcutaneous (s.c.) injection was less effective than intravenous injection; 10 μg/head was required to exhibit significant activity. Oral (p.o.) administration was also effective, although about 10 times FK565 was required to exhibit comparable activity to 1.0 μg/head administered i.v., i.p., and i.m. Sublingual (s.l.) administration was more effective than s.c., p.o., and intragingival (i.g.); significant effects were observed at 1.0 μg/head. Furthermore, intracerebroventricular injection of FK565 even at 0.01 μg/head exhibited significant activity; the required dose was only 1/100 as compared with that required for i.v., i.p., and i.m. routes.

Reduction of analgesic effects of FK565 by naloxone

Mice were injected subcutaneously with naloxone (a non-selective antagonist for opioid receptors), or saline, and 10 min later, the mice were injected intravenously with FK565 (10 μg/head) or saline. One hour later, the mice were subjected to the acetic acid test. The analgesic effects of FK565 were completely inhibited by 160 μg/head of naloxone (Fig. 5).

Analgesic effects of FK565 in Il-1α/β and/or TNF-α deficient mice

It was reported that IL-1α/β and TNF-α induced opioids[19,20] and exhibited analgesic activities in mice;[21] therefore, we examined whether FK565 exhibited analgesic effects in IL-1α/β knockout, TNF-α knockout and their triple knockout mice. As shown in Figure 6, FK565 exhibited significant analgesic effects in the three types of knockout mice similarly to wild-type mice. These findings clearly demonstrated that FK565 exhibited analgesic effects in IL-1α/β and TNF-α independent manners.

DISCUSSION

In this study, we demonstrated that both NOD1 and NOD2 agonists exhibited analgesic activity in terms of inhibiting writhing movements induced by acetic acid. In particular, NOD1 agonist FK565 exhibited marked analgesic activity; intravenous injections of 1.0 μg/head FK565 exerted stronger analgesic activity than 10 μg/head NOD2 agonist MDP (Fig. 2). The analgesic effect

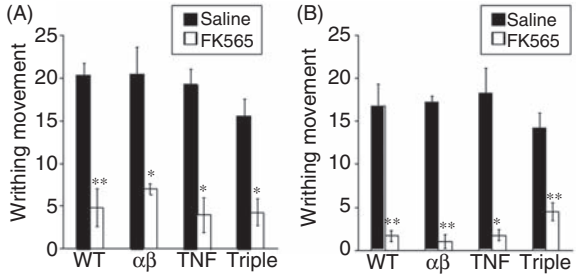

Fig. 6. Analgesic effects of FK565 in IL-1α/β knockout, TNF-α knockout and their triple knockout mice. The IL-1α/β knockout, TNF-α knockout and triple knockout mice (4 per group) were injected intravenously with FK565 (10 μg/head) or saline. One hour (A) or 8 h (B) later, the mice received an intraperitoneal injection of 1% acetic acid (0.2 ml/head), and the number of writhing movements was counted during 20 min. The results at 1 h and 8 h in wild-type mice are from Figures 2 and 3, respectively. $*P < 0.05$ and $**P < 0.01$ versus respective saline control.

of FK565 appeared from 30 min, peaked at 8 h, and continued until 24 h. Intravenous administration of MDP also exhibited a significant analgesic effect at 8 h as well as at 1 h (Fig. 3). On the other hand, Ogawa and Kotani[15] reported that intraperitoneal injection of MDP did not show analgesic activity at 8 h. Although the reason for the apparent discrepancies between their and our data is not clear at present, the administration route of the compound might be important. The FK565 was effective via various administration routes, not only the usual systemic routes, such as intravenous, intraperitoneal, intramuscular, and subcutaneous, but also oral routes, such as intragigival, sublingual and intragastric (Fig. 4). Furthermore, only 1/100 intravenous dose was effective when FK565 was administered intracerebrally. The analgesic activity of FK565 was completely inhibited by naloxone, a non-selective antagonist for opioid receptors (Fig. 5).[22] These findings suggested that NOD1 and NOD2 agonists induced opioids, which, in turn, exhibited an analgesic effect mainly on the central nervous system. In fact, a preliminary experiment suggested that intravenous injection of FK565 induced β-endorphin in blood (unpublished observation). Furthermore, MDP- or FK156-primed mice exhibited increased blood β-endorphin in response to intravenous injection of synthetic lipid A (unpublished observation). On the other hand, Horák and Mašek[16] reported that opioid receptor is not involved in the analgesic effect of MDP. The reason for the discrepancy between their and our findings is not clear at present. Further studies are in progress in our laboratory to elucidate which opioid receptor(s) is involved in the analgesic activity and which cell types are the main producers of opioids in response to NOD1/2 agonists.

Peptidoglycan is a ubiquitous structure on the bacterial cell surface whose active moieties possibly activate NOD2 and/or NOD1. Symbiosis with various bacteria is inevitable for mammals, including humans; therefore, mammals may receive continuous NOD2 and/or NOD1 stimulation. Bacterial endotoxin, which has a representative microbe-associated molecular pattern (MAMPS)[23] and is recognized by Toll-like receptor (TLR)4, was also reported to exert analgesic activity;[24] therefore, other TLR agonists might possess analgesic activity. In bacterial and microbial infections, hosts should be stimulated by NOD1/NOD2 and various TLRs, possibly resulting in decreased pain threshold.

In the future, it is expected that novel analgesic compounds mimicking bacterial components which simulate innate immune responses via NOD1/NOD2 or TLRs will be developed. In this study, among NOD1 agonists, FK565 was more than 10-times stronger than FK156 in terms of the dose to exert significant analgesic activity (Fig. 2), suggesting a possible a new drug design with powerful analgesic activity. In this context, oral administration, especially sublingual administration, is attractive.

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