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Research Article

Effect of Particle Size of Nonsteroidal Anti-Inflammatory Drug on Lipopolysaccharide-Induced Inflammation on RAW264.7 Cells

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Abstract

Background: Nonsteroidal anti-inflammatory drugs (NSAIDs) have excellent anti-inflammatory and analgesic effects and are widely used in inflammatory diseases. In this study, mouse macrophage cells stimulated with Lipopolysaccharide were used as a model experiment to investigate the influence of NSAIDs particle size on the inflammatory response.

Methods: RAW264.7 cells were used in this study. RAW264.7 cells, the survival rate was measured using the MTT method. Inflammation caused by using Lipopolysaccharide. The NSAIDs used were Indomethacin (IMC), ketprofen (KET), piroxicam (PXC), three kinds, and the anti-inflammatory effect on the bulk powder and nano particles was evaluated. Evaluation of inflammation was based on inflammatory cytokines (IL-6, TNF-a) and NO production.

Results: NSAID nano showed significant inhibitory effect on inflammatory cytokines (IL-6, TNF-a) and NO production compared with NSAIDbulk However, in NSAIDs with each particle size, no difference was found between the three drugs.

Conclusion: This suggests that the NSAIDnano preparation may be a raw material for new drugs. In addition, it was suggested that NSAIDnano preparation could reduce the concentration of the drug.

Keywords: Nonsteroidal antiinflammatory drugs (NSAIDs); Nanoparticles; RAW264.7 cells; Interleukin-6; Tumor necrosis factor-a; Nitric oxide; Prostagrandin; Cell culture

Introduction

Inflammatory reaction is a body defense reaction that occurs when foreign substances enter the body, or the body is exposed to noxious stimuli such as infection, injury, burns and allergens [1,2]. Although pain due to inflammatory reaction is one of the indispensable reactions that act as danger signals for the body, excessive inflammation has a bad influence on the human body, as it could cause damage and hypofunction of biological tissues and necessitate treatment with anti-inflammatory or other drugs [3]. Inflammatory reaction due to noxious stimuli resolves by repair involving microcirculatory changes, inflammatory cell infiltration and formation of granulation tissue. However, if the repair processes do not progress reasonably or local damage persists, inflammation can become chronic or prolonged. Chemical mediators produced by damaged tissues and inflammatory cells, such as nitric oxide (NO), histamine, prostaglandins (PGs), leukotrienes, platelet-activating factor, cytokines and cell growth factors, cause pain and dysfunction [1,2]. NO is a vascular smooth muscle relaxant factor produced by the vascular endothelial cells, and macrophages have the function of synthesizing NO: cytokines produced at sites of infection and inflammation, such as interleukin (IL)-1β, tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ), induce the expression of inducible nitric oxide synthase (iNOS), leading to the production of NO [4-6]. NO produced in excess reacts with superoxide to form peroxynitrite, which damages deoxyribonucleic acid (DNA), thereby damaging cells and tissues and exacerbating inflammatory symptoms. Nonsteroidal anti-inflammatory drugs (NSAIDs) have been widely used for the treatment of inflammatory diseases to suppress the inflammatory reactions described above. NSAIDs have excellent anti-inflammatory and analgesic effects and are widely used in the treatment of inflammatory diseases, such as chronic rheumatoid arthritis, and chronic musculoskeletal pain, such as low back pain and arthralgia, and for postoperative analgesia. NSAIDs inhibit the cyclooxygenases (COX-1 and COX-2), which play an important role in the conversion of arachidonic acid to eicosanoids [7,8]. Caution is required when NSAIDs are used for long periods of time, because the inhibition of COX-1 in the gastric mucosa causes gastrointestinal toxicity [9,10]. Recent studies have reported that NSAIDs suppress carcinogenesis in the stomach, colon, and other organs through inhibition of COX-2 [11], leading to the expansion of their use.

Nanotechnology is a technology that produces materials with new functions or excellent characteristics by controlling the structure and arrangement of materials at the nano level, and this technology is being actively developed [12]. At present, various nanomaterials are produced using this technology, and products in various fields, such as pharmaceuticals, foods, cosmetics, and chemicals, are already in the market. Nanomaterials are materials with a length of 1 to 100 nm or aggregates/clusters with nanoscale internal structures [13]. In the world of pharmaceuticals, advances in nanotechnology have allowed the development of drug delivery systems (DDS) using nanoparticles or microparticles as carriers. Drug systems using nanoparticles are expected as a strategy to increase drug bioavailability. Nanoparticles improve the bioavailability of orally administered NSAIDs and thereby reduce the required doses of these drugs, which may increase the use of nanoparticles in the treatment of patients with inflammatory diseases. Therefore, in this study, we investigated the influence of the particle size of NSAIDs on the viability of macrophages in the mouse. In addition, we also investigated the influence of the particle size of NSAIDs on the production of NO and inflammatory cytokines as a part of the inflammatory responses of these cells to stimulation with lipopolysaccharide (LPS).

Materials and Methods

Materials

Indomethacin (IMC), ketoprofen (KET), piroxicam (PXC) as bulk powder were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Other reagents used were guaranteed commercial-grade products.

Preparation of fine particles of NSAIDs

NSAIDs were incorporated into fine particles by processing the drug, zirconia beads (2.5 g), and an aqueous polymer (hydroxypropyl cellulose SSL [HPC]) solution in a planetary centrifugal mixer (NP-100; THINKY Corporation; fitted with a -20°C freezer) at a rotation rate of 1700 rpm, mixing time of 10 min, and medium (zirconia balls) quantity of 2.5 g. The particle size of the drug in the suspension was measured using a laser diffraction particle size distribution meter. The bulk powder (bulk) used in the present study were IMC (IMC_{bulk}), KET (KET_{bulk}), and PXC (PXC_{bulk}), and the fine-particle formulations of these drugs are expressed as IMC_{nano}, KET_{nano}, and PXC_{nano}, respectively.

Cell line and cell culture

RAW264.7 cells were used as the mouse macrophages. RAW264.7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL of penicillin and 100 µg/mL of streptomycin. The cells were cultured in a CO_2 incubator (37°C, 5% CO_2).

Evaluation of the toxicity of the NSAID nanoparticles on the mouse macrophages (RAW264.7 cells)

RAW264.7 cells were inoculated into 96-well plates at 1×10^5 cells/well and precultured for 24 hours. NSAIDs were added to the cells at final concentrations of 100, 50, 20 and 10 µg/mL, and the cells were cultured for 24 hours. A mixture of the cell suspension at 1×10^5 cells/well and culture medium was used as the control (CTL). After culture, the cells were stained with the MTT Cell Count Kit, and the optical density (OD) was measured using a microplate reader at 570 (630) nm. The OD of each sample relative to that of the CTL was calculated as the cell viability.

Evaluation of the anti-inflammatory effects of the NSAID nanoparticles on LPS-induced acute inflammation in the mouse macrophages (RAW264.7 cells)

RAW264 cells were inoculated into 96-well plates at 2×10^5 cells/well and precultured for 24 hours. LPS (100 ng/mL, lipopolysaccharide from *Escherichia coli* O127:B8, Sigma USA) and NSAIDs were added to the cells. The cells were cultured for 20 hours and the measurements were conducted in the culture supernatants. The NSAIDs were used at the final concentrations of 20 and 10 µg/mL. Culture medium was added instead of an NSAID in the control. The amounts of NO, inflammatory cytokines (IL-6 and TNF--α) and prostaglandin E2 were measured to evaluate the anti-inflammatory effects. The amount of NO production was assayed using the Griess method. TNF-α and IL-6 were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc., USA). Prostaglandin E2 was measured using a Prostaglandin E2 Assay Kit. Dexamethasone (DEX) was used as the positive control.

Statistical analysis

The obtained values are shown as the means ± standard error (SE). A one-way analysis of variance (ANOVA) with post hoc test was performed to determine the differences between the groups. The level of significance was 0.05.

Results

Formation of fine particles of NSAIDs

Fine particle formulations of IMC, KET and PXC were successfully created. However, diclofenac sodium could not be made into fine particles because it was soluble in the suspending agent. The particle sizes of the fine-particle NSAIDs are data not shown, the particle sizes were 72, 68 and 75 µm for IMCai, KETai and PXCai, respectively. Particle size measured 14 d post crushing, was unchanged from that recorded immediately after crushing.

Evaluation of the toxicity of the NSAID nanoparticles on the mouse macrophages

Table 1 shows the influence of the particle size of the NSAIDs on the mouse macrophages. The viability decreased in a dose-dependent manner for all NSAIDs. The $NSAID_{nano}$ decreased the viability of the RAW cells to a significantly greater degree than unprocessed $NSAID_{bulk}$ at concentrations of 50 and 100 µg/mL. However, at the concentrations of 10 and 20 µg/mL, there were no significant differences in the effect on the cell viability between the $NSAID_{nano}$ and unprocessed $NSAID_{bulk}$. Comparison among the NSAIDs revealed that PXC at a concentration of 100 g/mL was the most effective at reducing the viability of the RAW cells. The NSAIDs were used at the concentration of 20 µg/mL, at which there were no differences in their effects on the macrophage viability, to evaluate their anti-inflammatory effects.

Evaluation of the anti-inflammatory effects of the NSAID nanoparticles on the LPS-induced acute inflammatory responses in the mouse macrophages

The influence of NSAIDs on the production of NO in the mouse macrophages (RAW264.7 cells) induced by LPS was investigated. As shown in the Figure 1, the RAW 264.7 cells produced NO upon exposure to LPS. Both the unprocessed $NSAID_{bulk}$ and $NSAID_{nano}$ significantly suppressed the production of NO by the LPS-stimulated macrophages. $NSAID_{nano}$ suppressed the production of NO to a

		Concentration (µg/ml)	Cell Viability (%)
Bulk Powder	Indomethacin (IMC)	10	110.0 ± 1.9
		20	102.2 ± 2.3
		50	97.9 ± 2.7
		100	87.7 ± 2.0#,*
	Ketprofen (KET)	10	105.1 ± 0.3
		20	99.7 ± 2.3
		50	94.7 ± 2.1*
		100	87.2 ± 0.2#,*
	Piroxicam (PXC)	10	102.1 ± 0.5
		20	98.5 ± 2.1
		50	94.5 ± 3.2#,*
		100	86.1 ± 0.7#,*
Nano Particle	Indomethacin (IMC)	10	102.9 ± 1.5
		20	98.3 ± 1.3*
		50	89.7 ± 1.1#,*
		100	79.7 ± 0.6#,*
	Ketprofen (KET)	10	102.4 ± 2.0
		20	95.7 ± 3.1
		50	82.9 ± 1.0#,*
		100	77.8 ± 1.4#,*
	Piroxicam (PXC)	10	98.3 ± 3.6
		20	94.2 ± 3.0*
		50	85.6 ± 1.3#,*
		100	69.2 ± 1.6#,*

Each value is expressed as mean ± SE (n=3); #Statistically significant differences were seen when compared to the control (non-treated group) ($P<0.05$); *There were statistically significant differences when compared with the 20 µg/mL group ($P<0.05$)

Table 1: Cell viability of RAW cells after incubation with different NSAIDs for 24 h.

significantly greater degree than the unprocessed $NSAID_{bulk}$. Among the NSAIDs, IMC_{nano} most strongly suppressed the production of NO. Next, the influence of the NSAIDs on the production of inflammatory cytokines (IL-6 and TNF-α) induced by LPS was investigated (Figure 2). The influence of NSAIDs on IL-6 production is shown in Figure 2A. Both unprocessed $NSAID_{bulk}$ and $NSAID_{nano}$ significantly suppressed the production of IL-6, with the $NSAID_{nano}$ suppressed the production of IL-6 to a significantly greater degree than the unprocessed $NSAID_{bulk}$. Comparison among the $NSAID_{nano}$ revealed that the IL-6 production was significantly lower in the cells treated with IMC_{nano} than in those treated with the other $NSAID_{nano}$. Likewise, both unprocessed $NSAID_{bulk}$ and $NSAID_{nano}$ significantly suppressed the production of TNF-α, with the $NSAID_{nano}$ suppressed the production of TNF-α to a significantly greater degree than the unprocessed $NSAID_{bulk}$ (Figure 2B). Comparison among the $NSAID_{nano}$ showed that TNF-α production also was significantly lower in the cells treated with IMC_{nano} than in those treated with the other $NSAID_{nano}$. The influence of NSAIDs on the production of PGE2 is shown in Figure 3. Both unprocessed $NSAID_{bulk}$ and $NSAID_{nano}$ significantly suppressed the production of PGE2, with the $NSAID_{nano}$ suppressed the production of PGE2 to a significantly greater degree than the unprocessed $NSAID_{bulk}$. Comparison among the $NSAID_{nano}$ revealed that PGE2 production was significantly lower in the cells treated with IMC_{nano} than in those treated with the other NSAID nanoparticles.

Discussion

In this study, there was a difference between $NSAID_{bulk}$ and $NSAID_{nano}$ in all results. NSAIDs are reversible inhibitors of cyclooxygenase. They have excellent anti-inflammatory and analgesic effects and are widely used in the treatment of inflammatory diseases, such as chronic rheumatoid arthritis, and chronic musculoskeletal pain, such as low back pain and arthralgia, and for postoperative analgesia [14,15].

Drug systems using nanoparticles are expected as a strategy to increase drug bioavailability. Because nanoparticles improve the bioavailability and reduce the required doses of orally administered NSAIDs, their use in the treatment of patients with inflammatory diseases is expected to increase. In the present study, we prepared 3 types of $NSAID_{nano}$ and investigated the influence of differences in the particle size on the anti-inflammatory effects of these drugs on mouse macrophages, or RAW cells. The NSAIDs used in this study are also under consideration for nanoization in other reports. Our nanozoning method does not use special equipment. It is carried out using an easy-to-obtain rotating and revolving crusher (NP-100). Also, no organic solvent or stabilizer is added. In these respects, it is different from other reports and we believe it is an advantage of our method. Regarding the stability of $NSAID_{nano}$, it was confirmed the stability of the nanoparticles for 14 days (not show data) although it is not shown because it is being posted to another publication. In this study, NSAIDs showed dose-dependent cytotoxicity on the LPS-stimulated macrophage cell line (RAW 264.7); no cytotoxicity was observed at low concentrations.

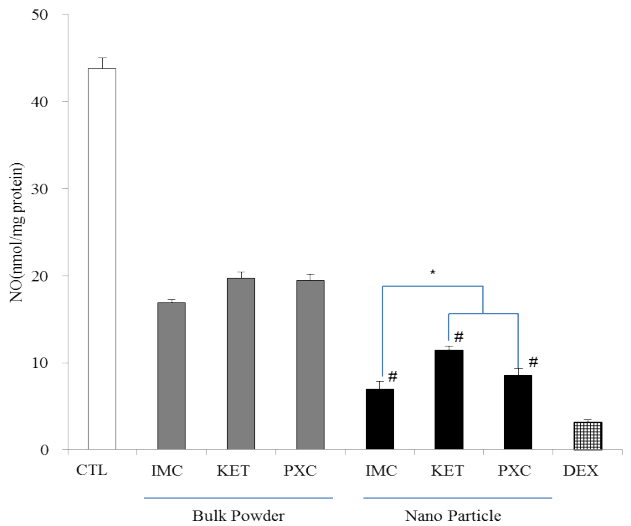

Figure 1: Effect of particle size on LPS-induced NO production of each NSAID in RAW264.7 cell. Each value is expressed as mean ± SE (n=3). Each NSAIDs group and DEX group showed a significantly lower value than the CTL group (not show mark). #Statistically significant differences were seen when compared to the each $NSAID_{bulk}$ (P<0.05). *There were statistically significant differences when compared with the IMC_{nano} group (P<0.05).

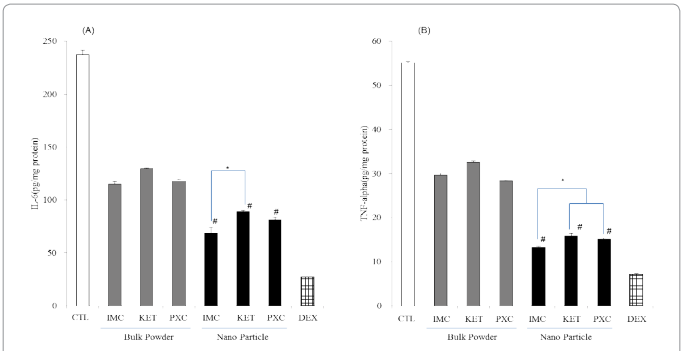

Figure 2: Effect of particle size on LPS-induced IL-6 (A) and TNF--α (B) production of each NSAID in RAW264.7 cell. Each value is expressed as mean ± SE (n=3). Each NSAIDs group and DEX group showed a significantly lower value than the CTL group(not show mark). #Statistically significant differences were seen when compared to the each $NSAID_{bulk}$ (P<0.05). *There were statistically significant differences when compared with the IMC_{nano} group (P<0.05).

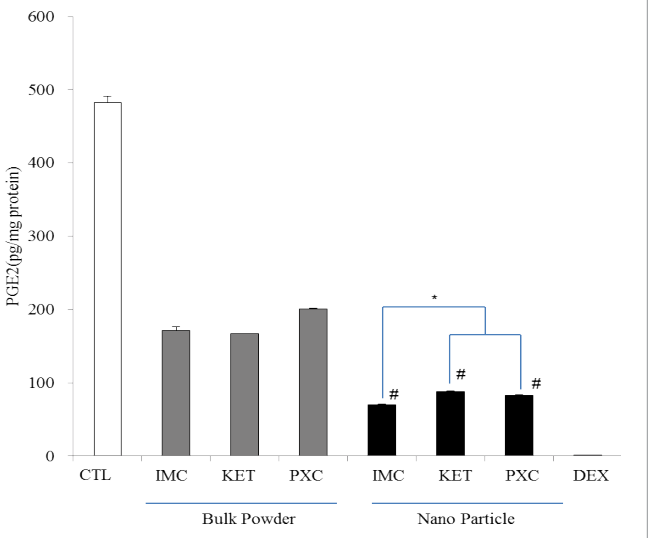

Figure 3: Effect of particle size on LPS-induced PGE2 production of each NSAID in RAW264.7 cell. Each value is expressed as mean ± SE (n=3). Each NSAIDs group and DEX group showed a significantly lower value than the CTL group (not show mark). #Statistically significant differences were seen when compared to the each $NSAID_{bulk}$ (P<0.05). *There were statistically significant differences when compared with the IMC_{nano} group (P<0.05).

In addition, the $NSAID_{nano}$ reduced the viability of the macrophages to a significantly greater degree than unprocessed $NSAID_{bulk}$ at high concentrations. A macrophage receptor with a collagenous structure expressed on the macrophage cell surface is reported to be involved in the phagocytosis of unopsonized particles [16], and also in the cellular binding and incorporation of nanoparticles [17]. Intracellular nanoparticles tend to accumulate in the mitochondria and are involved in the generation of reactive oxygen species and in the reduction of antioxidant activity, thereby causing cell damage [18-21]. Many $NSAID_{nano}$ at high concentrations were considered to be incorporated into the macrophages and to cause cell damage, thereby decreasing the viability of the cells. Therefore, the concentration of 20 μg/mL, at which there were no differences in the effects on the nanoparticles on the cell viability, was used to evaluate anti-inflammatory effects. Thus, the anti-inflammatory effects of the NSAIDs on the mouse macrophages in this study were unlikely to be affected by their cytotoxicity.

Evaluation of the anti-inflammatory effects of $NSAID_{nano}$ on LPS-induced acute inflammation in the mouse macrophages revealed that the NSAIDs suppressed the production of NO and inflammatory cytokines (TNF-α and IL-6) by the macrophages, and that the degree of the effect differed depending on the particle size of the $NSAID_{nano}$. The $NSAID_{nano}$ suppressed the production of NO and inflammatory cytokines to a significantly greater degree than the unprocessed $NSAID_{bulk}$. Among the NSAID nanoparticles, IMC_{nano} was the most effective at suppressing the production of NO, TNF-α and IL-6 by the macrophages. In addition, the $NSAID_{nano}$ also suppressed the production of prostaglandin E2 to a greater degree than the unprocessed $NSAID_{bulk}$. Among the NSAID nanoparticles, IMC_{nano} was the most effective at suppressing the production of prostaglandin E2. These results suggest that IMCnano exerted the strongest anti-inflammatory effects. As $NSAID_{nano}$ suppress the production of NO and other inflammatory cytokines to a significantly greater degree than unprocessed $NSAID_{bulk}$, they can be considered as having stronger anti-inflammatory effects. iNOS is induced by inflammation and stress [22,23]. During inflammation, a large amount of iNOS is expressed, with excessive production of NO, thereby damaging cells [24,25]. NO produced in the body has a variety of physiological activities, such as the regulation of vasorelaxation [26], neurotransmission [27] and infection [28]/inflammatory reactions [29]. On the other hand, NO has also been reported to be involved in carcinogenesis through its effects on DNA damage, angiogenesis and immunosuppression [29-32]. $NSAID_{nano}$ used in the present study suppressed the production of NO and PG to a much stronger degree than unprocessed $NSAID_{bulk}$, thereby exerting strong anti-inflammatory effects. however, the stronger suppression of NO and PG, which protect the gastrointestinal mucosa, production by $NSAID_{nano}$ than by unprocessed NSAIDs suggests that $NSAID_{nano}$ may exacerbate the adverse reactions to $NSAID_{bulk}$, especially gastrointestinal and renal disorders. $NSAID_{nano}$ used for this study showed a strong antiinflammatory effect by suppressing NO and PG production from the $NSAID_{bulk}$. This suggests that the $NSAID_{nano}$ preparation may be a raw material for new drugs. In addition, it was suggested that $NSAID_{nano}$ preparation could reduce the concentration of the drug. These facts can be considered to lead to miniaturization of preparations and suppression of medical expenses if the content of the drug is reduced. However, it is considered that the formulation containing NSAIDs nanoparticles may change the *in vivo* dynamics and safety, and it is considered that the same evaluation as new drugs is necessary for use. In the future, detailed animal studies are required on the influence of NSAID nanoparticles on the gastrointestinal tract and kidney, the optimal route of administration of these particles, etc.

Conflict of Interest

The authors declare that they have no conflict of interest to disclose.

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