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Inhibitory Effect of Probenecid on Osteoclast Formation via JNK, ROS and COX-2

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Abstract

Probenecid is a representative drug used in the treatment of gout. A recent study showed that probenecid effectively inhibits oxidative stress in neural cells. In the present study, we investigated whether probenecid can affect osteoclast formation through the inhibition of reactive oxygen species (ROS) formation in RAW264.7 cells. Lipopolysaccharide (LPS)-induced ROS levels were dose-dependently reduced by probenecid. Fluorescence microscopy analysis clearly showed that probenecid inhibits the generation of ROS. Western blot analysis indicated that probenecid affects two downstream signaling molecules of ROS, cyclooxygenase 2 (COX-2) and c-Jun N-terminal kinase (JNK). These results indicate that probenecid inhibits ROS generation and exerts antiosteoclastogenic activity by inhibiting the COX-2 and JNK pathways. These results suggest that probenecid could potentially be used as a therapeutic agent to prevent bone resorption.

Key Words: Probenecid, Antiosteoclastogenesis, Oxidative stress, ROS, COX-2, JNK

INTRODUCTION

Osteoblasts and osteoclasts work together to control the amount of total bone mass by regulating bone remodeling. It has been suggested that pathological bone diseases such as rheumatoid arthritis and osteoporosis are caused by excessive osteoclast differentiation (Boyle *et al*., 2003). Receptor activator of nuclear factor kappa-B ligand (RANKL) plays an important role in osteoclast activation and differentiation (Yasuda *et al*., 1998; Suda *et al*., 1999; Teitelbaum and Ross, 2003). Various signaling molecules including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), extracellular signal-regulated kinase (ERK), PI3K/Akt, c-Jun N-terminal kinase (JNK) and cyclooxygenase 2 (COX-2) are involved in osteoclastogenesis (Darnay *et al*., 1999; Matsumoto *et al*., 2000; Zhang *et al*., 2001; Hou *et al*., 2013; Mizutani *et al*., 2013; Chen *et al*., 2019).

Cell damage due to oxidative stress is associated with a diverse range of metabolic diseases including osteoporosis, diabetes and neurodegenerative disorders like Alzheimer's disease. Reactive oxygen species (ROS) are the most important mediator of cell damage caused by oxidative stress. The ROS include superoxide radical (O_2^-), hydroxyl radical (OH•), hydrogen peroxide (H_2O_2) and peroxynitrate ($ONOO^-$), which occur when DNA damage, oxidative burst, cell lysis, protein oxidation, lipid peroxidation, excitatory amino acids and cell death including apoptosis (Chandra *et al*., 2000; Ruffels *et al*., 2004; Zhang *et al*., 2007; Zhuang *et al*., 2007; Chen *et al*., 2009; Lin *et al*., 2009). Various cytokines and growth factors, such as TNF-α, are known to bind to receptors to produce ROS, which triggers an increase in ROS levels in a variety of cells (Thannickal and Fanburg, 2000). High levels of ROS in cells can lead to inflammatory reactions, aging, apoptosis and cancer. In contrast, low levels of ROS can serve as secondary messengers in various signaling pathways (Sundaresan *et al*., 1995; Bae *et al*., 1997; Forman *et al*., 2004). Studies have been conducted on the role of ROS in osteoclast differentiation (Lee *et al*., 2005). Osteoclasts have been found to be highly susceptible to oxidative stress, and they are activated and differentiated by ROS (Garrett *et al*., 1990; Steinbeck *et al*., 1994; Fraser *et al*., 1996).

Probenecid has long been used as a treatment for gout. It inhibits the renal tubular transporter to prevent the reuptake of uric acid, thereby promoting the excretion of uric acid (Gutman, 1951; Beachwood *et al*., 1964; Moller, 1965). In a recent study, probenecid was shown to exhibit antihypertensive

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action by inhibiting the α-adrenergic receptor (Park and Kim, 2011). Furthermore, it was found that probenecid blocks the efflux of antioxidant glutathione (GSH), as well as GSH conjugates in neurons (Du *et al*., 2016). Although the molecular mechanisms involved in oxidative stress-induced osteoclast differentiation are complex and not well characterized (Lee *et al*., 2005; Oka *et al*., 2012), therapeutic strategies to prevent ROS generation may be useful in overcoming many diseases, including osteoporosis and diabetes. Here, we investigate whether probenecid has the potential to regulate osteoclastogenesis by inhibiting oxidative stress. The objective of this study was to evaluate whether probenecid inhibits lipopolysaccharide (LPS)-induced osteoclast formation by inhibiting ROS production using RAW264.7 cells.

MATERIALS AND METHODS

Reagents and materials

Ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA), sodium azide, LPS and 2,7-dichlorofluorescin diacetate (DCF-DA) were purchased from Sigma Chemical Co (St. Louis, MO, USA). The materials needed for cell culture were obtained from Gibco BRL (Gaithersburg, MD, USA), and VECTASHIELD mounting medium for fluorescence staining with DAPI was purchased from Vector Laboratories Inc (Burlingame, CA, USA). JNK, COX-2 and GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The ECL kit was purchased from Amersham BioScience (Amersham, UK) and probenecid was obtained from Sigma Chemical Co.

Cell culture

The RAW264.7 cell line was purchased from American Type Culture Collection (ATCC; ATCC®TIB-71, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, NY, USA) containing 10% fetal bovine serum (FBS; Gibco), penicillin (100 U/mL; Gibco) and streptomycin (100 μg/mL; Gibco) in a humidified incubator with 5% CO_2 at 37°C. Cells were cultured in a six-well plate at a density of 10^6 for 24 h prior to probenecid treatment. After cells had grown to confluence, they were incubated in a serum-free starvation state. Serum-free starvation cells were incubated with probenecid at four different concentrations (0.1, 1, 10 and 100 μM) for 4 h, then treated with LPS (10 μg/mL) for 24 h. The cells were subjected to western blot analysis.

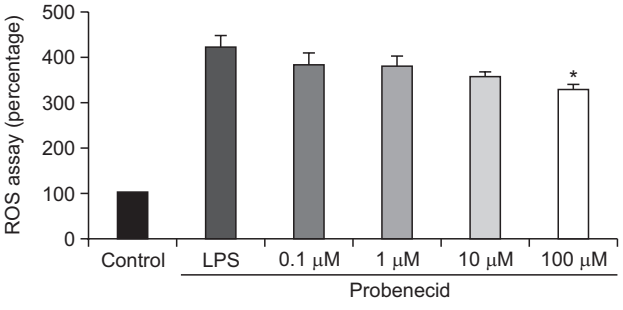

Fig. 1. Probenecid inhibits lipopolysaccharide (LPS)-induced reactive oxygen species (ROS) production in RAW264.7 cells. RAW264.7 cells grown in serum-free medium were incubated with probenecid (0.1, 1, 10 and 100 μM) for 4 h, then cultured for 24 h after LPS (10 μg/mL) treatment. Measurement of ROS in cells was performed using a microplate reader, as described in the "Materials and Methods" section. Data are presented as the mean ± standard deviation of four experiments performed in triplicate. *p<0.05 compared to the LPS-treated group.

Detection of intracellular reactive oxygen species

Intracellular ROS were detected using 2,7-dichlorofluorescin diacetate (DCF-dAc), a membrane-permeable tracer that is oxidized by various types of ROS. For this, 100 μM of DCF-dAc was added to cells that had been cultured as described above, and incubated in the dark for 30 min at 37°C. The cells were washed twice with phosphate-buffered saline (PBS). Fluorescence was measured using a fluorescent microplate reader (TRIAD Series Multimode Detector; Dynex Technologies, Inc., VA, USA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Intracellular reactive oxygen species staining

To assess intracellular ROS staining, 100 μM of DCFH-DA was added to cells cultured as described above, and incubated in the dark for 30 min at 37°C. The cells were washed three times with PBS then mounted on glass slides using aqueous mounting medium containing DAPI. DCF and DAPI fluorescence were detected using an Olympus BX50 microscope (Olympus, Tokyo, Japan) and images were acquired using an Olympus DP71 camera (Olympus).

Western blot analysis

The cells were washed three times with PBS, scraped off, then centrifuged at 12,000 rpm for 5 min. Cells were collected and lysed as previously described (Jang and Kim, 2013). The concentration of total protein was quantified using a Bradford assay (Bio-Rad, Hercules, CA, USA). Samples with equal amounts of protein (50 μg) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) then transferred to a nitrocellulose membrane. To prevent

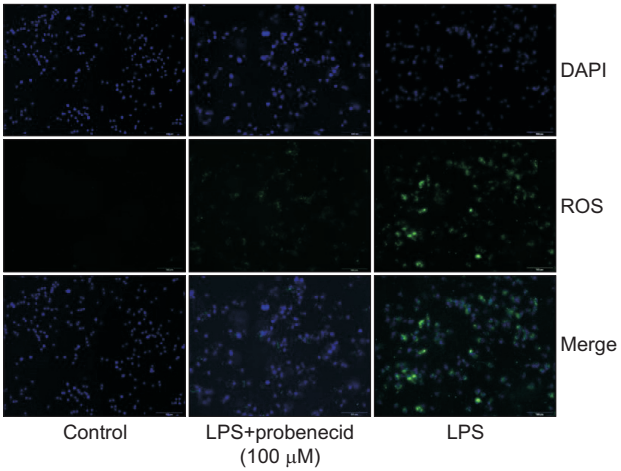

Fig. 2. Detection of intracellular reactive oxygen species (ROS) induced by lipopolysaccharide (LPS) in the presence of probenecid in LPS-treated RAW264.7 cells. RAW264.7 cells grown in serum-free medium were incubated with probenecid (0.1, 1, 10 and 100 μM) for 4 h, then cultured for 24 h after LPS (10 μg/mL) treatment. Assessment of ROS by microscopy was performed as described in the "Materials and Methods" section.

non-specific binding, membranes were incubated in 5% skim milk for 1 h. After blocking, each membrane was incubated with antibodies against pJNK (1:1,000; Santa Cruz Biotechnology), COX-2 (1:2,000; Santa Cruz Biotechnology) and GAPDH (1:1,000; Santa Cruz Biotechnology). The blots were detected using a chemiluminescence detection system (ECL, Amersham BioScience).

Ileum contractile analysis

The contraction of rat ileum was measured as previously described (Schapoval *et al*., 1998) but with slight modifications. The contraction response was constantly recorded in an isotonic solution using Grass Low Level DC Amplifier (model 7P122P). After 30 min of equilibration, concentration-response curves were generated by placing the contractile agonist (bradykinin, 10 μM) in the organ bath. After washing the ileum and restoring baseline tension, probenecid (1 μM) was added to the organ bath and incubated for 15 min. Bradykinin was added and the contraction reaction was recorded using MacLab 8E data acquisition system (AD Instruments, Vella Vista, New South Wales, Australia).

Tartrate-resistant acid phosphatase assay

After culturing cells according to the above method and washing with PBS, a tartrate-resistant acid phosphatase (TRAP) assay was performed according to the manufacturer's instructions (Sigma Chemical Co.). AP assay buffer was added to the cells and incubated at 37°C for 2 h. The AP stop buffer was added to stop the reaction, and the absorbance was read at 410 nm with a TRIAD Series Multimode Detector (Dynex Technologies Inc.).

Statistical analysis

All experiments were performed in triplicate, and the results are expressed as the mean ± standard deviation. Data were analyzed using GraphPad Prism 5 software (GraphPad software, La Jolla, CA, USA). One-way ANOVA and Tukey's HSD post hoc test were used. Differences were considered statistically significant at $p<0.05$.

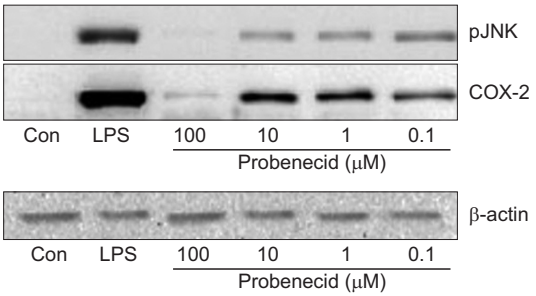

Fig. 3. Western blot analysis of pJNK and COX-2 in response to probenecid in lipopolysaccharide (LPS)-treated RAW264.7 cells. RAW264.7 cells grown in serum-free medium were incubated with probenecid (0.1, 1, 10 and 100 μM) for 4 h, then cultured for 24 h after LPS (10 μg/mL) treatment. The western blot procedure is described in the "Materials and Methods" section.

RESULTS

Probenecid inhibits lipopolysaccharide-induced production of reactive oxygen species in RWA264.7 cells

Untreated RAW264.7 cells showed increased LPS-induced ROS production, while probenecid treatment suppressed ROS production in a dose-dependent manner (Fig. 1). The fluorescence analysis confirmed that LPS-induced ROS production was reversed by probenecid (Fig. 2). The concentration of probenecid with the greatest ROS inhibition was 100 μM.

Effect of probenecid on signaling molecules downstream of reactive oxygen species

To investigate the mechanism by which probenecid inhibits the ROS induced by LPS, western blot analysis was performed using antibodies specific to JNK and COX-2. While the phosphorylation of JNK (pJNK) was dramatically induced by LPS, it was markedly decreased when pretreated with probenecid, and the maximum inhibitory concentration of probenecid was 100 μM (Fig. 3).

Effect of probenecid on bradykinin-mediated rat ileum contraction

Isolated rat ileum is a sensitive bioassay tissue for testing biomolecules with antagonistic effects on the bradykinin B1 receptor (Vietinghoff *et al*., 2003), therefore we assessed whether probenecid could affect the binding of bradykinin to its B1 receptor by employing rat ileum contraction experiments. Probenecid did not show any effect on bradykinin binding (Fig. 4).

Probenecid inhibits osteoclast formation in RAW264.7 cells

To confirm whether probenecid affects LPS-induced osteoclastogenesis, a TRAP assay was performed. Probenecid significantly inhibited LPS-induced osteoclast differentiation, and the highest inhibitory concentration was 100 μM (Fig. 5).

DISCUSSION

Probenecid has been studied for many decades and is known to exhibit various pharmacological actions. In this study, we examined whether probenecid could affect osteoclastogenesis and whether ROS, JNK and COX-2 are involved

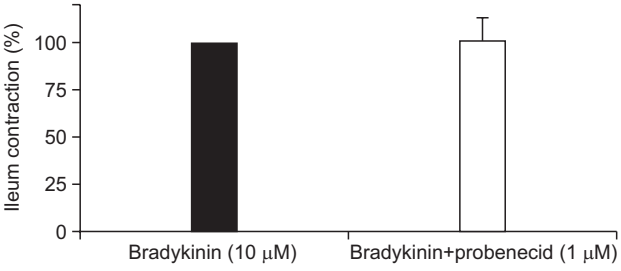

Fig. 4. Effect of probenecid on bradykinin-induced rat ileum contraction. Segmented rat ileum was placed in an organ bath with a load of 1 g at 37°C. The ileum was equilibrated for 30 minutes before the experiment began. Organ processing for the ileum contraction assay is described in the "Materials and Methods" section.

in the action of probenecid. Clinical studies have shown that probenecid is effective in reducing serum uric acid levels. It has been used as a treatment for gout for decades, and has been shown to improve the symptoms of gout patients (Talbott, 1951; Sirota *et al*., 1952; Bishop and Pfaff, 1955; Boger and Strickland, 1955).

In addition to increasing uric acid excretion, probenecid inhibits the α-adrenergic receptor, exhibiting an antihypertensive effect. Interestingly, probenecid also interferes with the efflux of GSH and GSH conjugates in neurons, suggesting the possibility of an antioxidative effect on osteoclast differentiation. Therefore, it is reasonable to assume that the ability of probenecid to interfere in oxidative stress could mean that it also affects osteoclast differentiation. We present evidence that probenecid inhibits osteoclastogenesis by inhibiting JNK phosphorylation, ROS production and COX-2 expression. It is well known that intracellular ROS are generated during the process of osteoclastogenesis. The increased level of ROS also promotes osteoclastogenesis and plays an important role in the signaling process involved in osteoclast activation, resulting in bone resorption (Hall *et al*., 1995). In the present study, we provide evidence that probenecid inhibits LPS-induced ROS production in RAW264.7 cells, suggesting that ROS could be a target of probenecid action. However, the reduction in ROS production by probenecid was not dramatic, indicating that it might play a minor role in the inhibition of osteoclastogenesis.

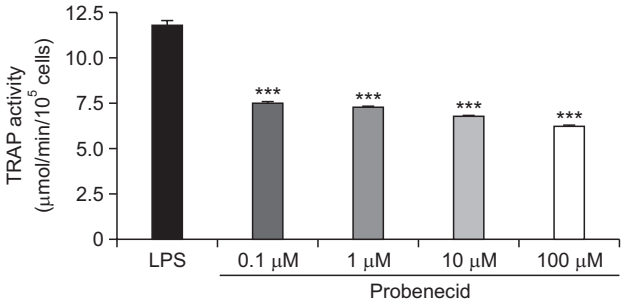

Fig. 5. Probenecid blocks osteoclastogenesis in RAW264.7 cells. RAW264.7 cells grown in serum-free medium were incubated with probenecid (0.1, 1, 10 and 100 μM) for 4 h, then cultured for 24 h after LPS (10 μg/mL) treatment. The subsequent TRAP assay procedure is described in the "Materials and Methods" section. Data are presented as the mean ± standard deviation of four experiments performed in triplicate. ***p<0.001 compared to the LPS-treated group.

Considering that ROS requires an activated JNK, we attempted to determine whether JNK activity could be influenced by probenecid. As shown in Fig. 3, LPS-induced phosphorylation of JNK was significantly reduced by probenecid, indicating that it is an important molecular target in the antiosteoclastogenic activity of probenecid. LPS has been reported to increase osteoclastogenesis through the activation of JNK and increased expression of COX-2 (Hou *et al*., 2013), suggesting that JNK phosphorylation and COX-2 expression could be essential regulators of osteoclast differentiation.

In this study, we examined the effect of probenecid on the COX-2 signaling pathway. As shown in Fig. 3, the LPS-induced expression of COX-2 was inhibited by probenecid at concentrations ranging from 0.1 to 100 μM. However, there was a small increase in the expression of COX-2 in cells treated with 1 to 10 μM of probenecid when compared to those treated with 0.1 μM. COX-2 is a rate-limiting enzyme for prostaglandin synthesis, and also undergoes feedback control depending on changes in its expression (Tang *et al*., 2017). It is possible that probenecid at concentrations of 0.1 to 10 μM may have some effect on the signaling mediators involved in the feedback loop of COX-2 expression, thereby inducing slight increases in COX-2 expression under LPS treatment as the concentration increases from 0.1 to 10 μM. Nevertheless, probenecid inhibits LPS-induced COX-2 expression, supporting its antiosteoclastogenic action.

Bradykinin has been found to increase mitochondrial ROS production in cell culture (Greene *et al*., 2000; Oldenburg *et*

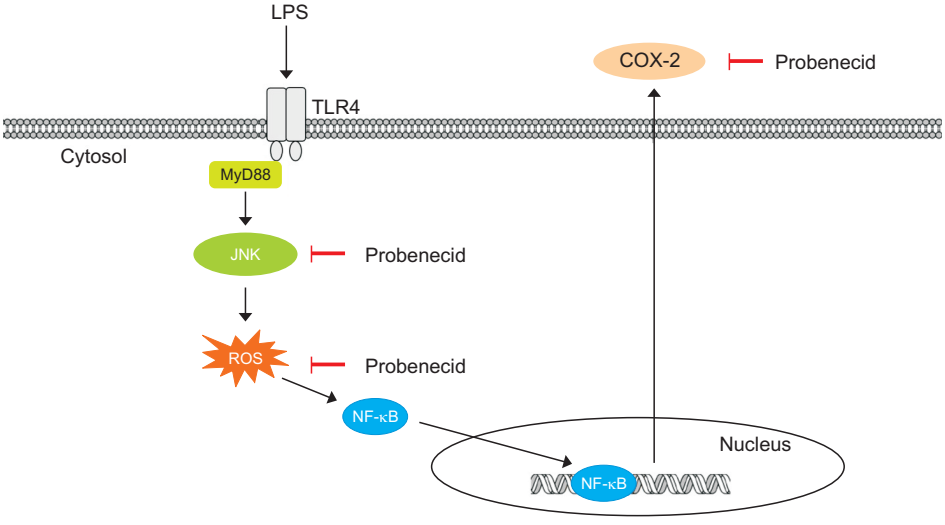

Fig. 6. Schematic illustration of the proposed mechanism of probenecid in lipopolysaccharide (LPS)-induced RAW264.7 cells. Probenecid exhibits antiosteoclastogenic activity by inhibiting reactive oxygen species (ROS) formation, JNK phosphorylation and COX-2 expression.

al., 2004), inducing inflammation. Bradykinin receptors (B1 and B2) are expressed in smooth muscle cells, and bradykinin has a higher affinity for the B1 receptor than the B2 receptor. B2 receptors are constitutively expressed in many tissues whereas B1 receptors are induced under conditions of tissue injury, such as inflammation and oxidative stress (Vietinghoff *et al*., 2003). Isolated rat ileum is widely used to investigate potential antagonistic effects on the bradykinin B1 receptor. In the present study, probenecid had no effect on bradykinin-induced rat ileum contraction, suggesting that probenecid may not directly interfere in the binding of bradykinin to B1 receptors. A TRAP assay was performed to confirm that RAW264.7 cells were differentiated into osteoclasts by LPS. Treatment with probenecid blocked osteoclast differentiation, confirming that probenecid inhibited osteoclast differentiation (Fig. 5). A recent study showed that the COX-2 inhibitor, celecoxib, inhibits RANK expression, and thus inhibits osteoclast differentiation (Geng *et al*., 2011). Therefore, it is reasonable to assume that probenecid inhibits osteoclast formation by inhibiting COX-2 and RANK expression. We took all measurents 24 h after stimuli, since we and other investigatiors have previously found that ROS production and JNK activation by LPS could last for 24 h (Jang and Kim, 2013; Hwang *et al*., 2016). Considering the early activation of JNK, we depicted a signaling cascade of JNK - ROS -NFκB - COX2 in Fig. 6. Our proposed mechanism of action is shown in Fig. 6. It will be necessary to explore whether other signaling proteins that are important for osteoclastogenesis, such as cathepsin K, calcitonin receptor, TNF receptor-associated factor 6 and matrix metalloproteinase 9, are involved in the action of probenecid.

In conclusion, we found an effect of probenecid on osteoclast formation, and the mechanism of action of probenecid may involve the inhibition of JNK phosphorylation, ROS formation, and the subsequent blocking of COX-2 expression. Currently available drugs for the treatment of metabolic bone diseases include bisphosphonates, denosumab and selective estrogen receptor modulators. However, these drugs have serious side effects such as osteonecrosis of the jaws and increased endometrial cancer risk (Wu *et al*., 2018; Zhang *et al*., 2018), highlighting the necessity for novel drug development. Taken together, our results suggest that probenecid could potentially be used as a new therapeutic agent for the treatment of metabolic bone diseases such as osteoarthritis, osteoporosis and rheumatic arthritis.

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