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

Mi Hyun Cheng and Sung-Jin Kim*

Department of Pharmacology and Toxicology, School of Dentistry, Graduate School, Kyung Hee University, Seoul 02447, Republic of Korea

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
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 (Na₂-EDTA), sodium azide, LPS and 2,7-dichlorofluorescin diacetate (DCF-DNA) 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% CO₂ at 37°C. Cells were cultured in a six-well plate at a density of 10⁶ for 24 h prior to probenecid treatment. After cells had grown to confluence, they were incubated in 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.

**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.

**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. Measurement of ROS by microscopy was performed as described in the “Materials and Methods” section. Assessment of ROS by microscopy was performed by staining cells with DCFH-DA (1 μM) for 10 min, then coincubated with LPS (10 μg/mL) for 24 h. Fluorescence was measured using a fluorometer with excitation at 485 nm and emission at 530 nm. The data are presented as the mean ± standard deviation of four experiments performed in triplicate. *p<0.05 compared to the LPS-treated group.

**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 nonspecific binding, membranes were blocked with 5% nonfat dry milk in Tris-buffered saline-Tween 20 (TBS-T) for 1 h. Membranes were then incubated with anti-osteoclastogenicity antibodies overnight at 4°C. Membranes were then washed with TBS-T three times for 5 min each and incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000, Santa Cruz Biotechnology) for 1 h at room temperature. Membranes were washed with TBS-T three times for 5 min each, and visualized with 4-chloro-1-naphthol (Sigma) and hydrogen peroxide (30%) as substrates. The protein bands were analyzed by densitometry using Image J software.
Probenecid inhibits lipopolysaccharide-induced production of reactive oxygen species in RAW264.7 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 in this process.
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.

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 10 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 al., 2012).

![Fig. 5](image_url) 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.

![Fig. 6](image_url) 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.
REFERENCES

Bae, Y. S., Kang, S. W., Seo, M. S., Baines, I. C., Tekle, E., Chock, P. B. and Rhee, S. G. (1997) Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. J. Biol. Chem. 272, 217-221.

Beachwood, E. C., Bermdt, W. O. and Mudge, G. H. (1964) Stop-flow analysis of tubular transport of uric acid in rabbits. Am. J. Physiol. 207, 1265-1272.

Bishop, C. and Pfaff, W. (1955) Immediate uricosic effect of probenecid in normal humans. Proc. Soc. Exp. Biol. Med. 88, 346-348.

Boger, W. P. and Strickland, S. C. (1955) Probenecid (benemid); Its mechanism of action. J. Clin. Invest. 34, 280-287.

Hou, G. Q., Guo, C., Song, G. H., Fang, N., Fan, W. J., Chen, X. D., Yuan, L. and Wang, Z. Q. (2013) Lipopolysaccharide (LPS) promotes osteoclast differentiation and activation by enhancing the MAPK pathway and COX-2 expression in RAW264.7 cells. Int. J. Mol. Med. 32, 503-510.

Hwang, J. H., Kim, S. J. and Bae, Y. S. (2011) Taurine exerts anti-osteoclastogenesis activity via inhibiting ROS generation, JNK phosphorylation and COX-2 expression in RAW264.7 cells. J. Recept. Signal Transduct. Res. 31, 387-391.

Lee, N. K., Choi, Y. G., Balik, J. Y., Han, S. Y., Jeong, D. W., Bae, Y. S., Kim, N. and Lee, S. Y. (2005) A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation. Blood 106, 852-859.

Lin, Y. C., Huang, Y. C., Chen, S. C., Liaw, C. C., Kuo, S. C., Huang, L. J. and Gean, P. W. (2009) Neuroprotective effects of Ugonin on hydrogen peroxide-induced cell death in human neuroblastoma SH-SY5Y cells. Neurochem. Res. 34, 923-930.

Matsumoto, M., Sudo, T., Saito, T., Osada, H. and Tsuchiya, T. (2000) Involvement of p38 mitogen-activated protein kinase signal transduction pathway in osteoclastogenesis mediated by receptor activator of NF-kappa B ligand (RANKL). J. Biol. Chem. 275, 31155-31161.

Mizutani, H., Ishiihara, Y., Izawa, F., Fujihara, Y., Kobayashi, S., Goto, H., Okabe, E., Takeda, H., Otsuka, Y., Kamiya, Y., Kamei, H., Kikuchi, T., Yamamoto, G., Mitani, A., Nishihara, T. and Naguchi, T. (2013) Lipopolysaccharide of Aggregatibacter actinomycetem-
comitans up-regulates inflammatory cytokines, prostaglandin E2 synthesis and osteoclast formation in interleukin-1 receptor antagonist-deficient mice. J. Periodont. Res. 48, 748-756.

Moller, J. V. (1965) The tubular site of urate transport in the rabbit kidney, and the effect of probenecid on urate secretion. Acta Pharmacol. Toxicol. (Copenh.) 23, 329-336.

Oka, Y., Iwai, S., Amano, H., Irie, Y., Yatomi, K., Ryu, K., Yamada, S., Inagaki, K. and Oguchi, K. (2012) Tea polyphenols inhibit rat osteoclast formation and differentiation. J. Pharmacol. Sci. 118, 55-64.

Oldenburg, I., Qin, Q., Krieg, T., Yang, X. M., Philipp, S., Critz, S. D., Cohen, M. V. and Downey, J. M. (2004) Bradykinin induces mitochondrial ROS generation via NO, cGMP, PKG, and mitoKATP channel opening and leads to cardioprotection. Am. J. Physiol. Heart Circ. Physiol. 286, H468-H476.

Park, J. B. and Kim, S. J. (2011) Anti-hypertensive effects of probenecid via inhibition of the α-adrenergic receptor. Pharmacol. Rep. 63, 1145-1150

Ruffels, J., Griffin, M. and Dickenson, J. M. (2004) Activation of ERK1/2, JNK and PKB by hydrogen peroxide in human SH-SYSY neuroblastoma cells: Role of ERK1/2 in H2O2-induced cell death. Eur. J. Pharmacol. 483, 163-173.

Schapoval, E. E., Vargas, M. R., Chaves, C. G., Bridi, R., Zuanazzi, J. A. and Henriques, A. T. (1998) Antiinflammatory and antinociceptive activities of extracts and isolated compounds from Stachytarpheta cayennensis. J. Ethnopharmacol. 60, 53-59.

Sirote, J. H., Yu, T. F. and Gutman, A. B. (1952) Effect of benemid (p-[di-n-propylsulfamyl]-benzoic acid) on urate clearance and other discrete renal functions in guinea subjects. J. Clin. Invest. 31, 692-701.

Steinbeck, M. J., Appel, W. H., Jr., Verhoeven, A. J. and Karmovsky, M. J. (1994) NADPH-oxidase expression and in situ production of superoxide by osteoclasts actively resorbing bone. J. Cell Biol. 126, 765-772.

Suda, T., Takahashi, N., Udagawa, N., Jimi, E., Gillespie, M. T. and Martin, T. J. (1999) Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr. Rev. 20, 345-357.

Sundaresan, M., Yu, Z. X., Ferrans, V. J., Irani, K. and Finkel, T. (1995) Requirement for generation of H2O2 for platelet-derived growth factor signal transduction. Science 270, 296-299.

Talbott, J. H. (1951) Clinical and metabolic effects of benemid in gout. Bull. Rheum. Dis. 2, 1-2.

Tang, T., Scambler, T. E., Smallie, T., Cunliffe, H. E., Ross, E. A., Rosser, D. R., O’Neil, J. D. and Clark, A. R. (2017) Macrophage responses to lipopolysaccharide are modulated by a feedback loop involving prostaglandin E2, dual specificity phosphatase 1 and tristetraprolin. Sci. Rep. 7, 4350.

Teitelbaum, S. L. and Ross, F. P. (2003) Genetic regulation of osteoclast development and function. Nat. Rev. Genet. 4, 638-649.

Thannickal, V. J. and Fanburg, B. L. (2000) Reactive oxygen species in cell signaling. Am. J. Physiol. Cell Physiol. 279, L1005-L1028.

Vieteinhoff, G., Hilscher, E., Paegelow, I. and Reissmann, S. (2003) Effect of Bradykinin analogues on the B1 receptor of rat ileum. Peptides 24, 931-935.

Wu, H., Hu, B., Zhou, X., Zhou, C., Meng, J., Yang, Y., Zhao, X., Shi, Z. and Yan, S. (2018) Artemether attenuates LPS-induced inflammatory bone loss by inhibiting osteoclastogenesis and bone resorption via suppression of MAPK signaling pathway. Cell Death Dis. 9, 498.

Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S., Tomoyasu, A., Yano, K., Goto, M., Murakami, A., Tsuda, E., Morinaga, T., Higashio, K., Udagawa, N., Takahashi, N. and Suda, T. (1998) Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc. Natl. Acad. Sci. U.S.A. 95, 3597-3602.

Zhang, L., Yu, H., Sun, Y., Lin, X., Chen, B., Tan, C., Cao, G. and Wang, Z. (2007) Protective effects of salodroside on hydrogen peroxide-induced apoptosis in SH-SYSY human neuroblastoma cells. Eur. J. Pharmacol. 564, 18-25.

Zhang, X., Li, X., Fang, J., Hou, X., Fang, H., Guo, F., Li, F., Chen, A. and Huang, S. (2018) (2R,3R)Dihydromyricetin inhibits osteoclastogenesis and bone loss through scavenging LPS-induced oxidative stress and NF-κB and MAPKs pathways activating. J. Cell. Biochem. 119, 8981-8995.

Zhang, Y. H., Heulsmann, A., Tondravi, M. M., Mukherjee, A. and Abu-Amer, Y. (2001) Tumor necrosis factor-alpha (TNF) stimulates RANKL-induced osteoclastogenesis via coupling of TNF type 1 receptor and RANK signaling pathways. J. Biol. Chem. 276, 563-568.

Zhuang, S., Yan, Y., Daubert, R. A., Han, J. and Schnellmann, R. G. (2007) ERK promotes hydrogen peroxide-induced apoptosis through caspase-3 activation and inhibition of Akt in renal epithelial cells. Am. J. Renal Physiol. 292, 440-447.