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Nicorandil inhibits osteoclast differentiation in vitro

Futoshi Iwaki, Hitoshi Amano*, Kiyoshi Ohura

Department of Pharmacology, Osaka Dental University, -1, Kuzuhahanazono-cho, Hirakata-shi 573-1121, Japan

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ABSTRACT

Nicorandil is a hybrid angina therapeutic agent that has nitric oxide (NO) action and the ability to open ATP-sensitive K^+ channels (K_{ATP} channels). A transient increase in NO and intracellular Ca^{2+} has been demonstrated to be highly involved in the differentiation and activation of osteoclasts. The objective of this study was to verify that the pharmacological effect of nicorandil suppresses the differentiation process of osteoclasts in vitro.

Although little authentic NO production was detected in the culture medium in osteoclast formation assays, NO production increased only in the presence of nicorandil. The number of osteoclasts decreased markedly at late time-points after nicorandil addition compared with the number at early time-points. Both the number of TRAP-positive multinucleated cells and the number of cells that obtained F-actin rings decreased in the presence of nicorandil in a concentration-dependent manner. The osteo assay showed that the bone resorption area was also reduced with nicorandil in a concentration-dependent manner. An inhibition recovery experiment was conducted by adding a soluble guanylyl cyclase (sGC) inhibitor (ODQ) and a K_{ATP} channel-opening inhibitor (glibenclamide) during the osteoclast formation process. In the inhibition recovery experiment, the inhibitory effect of nicorandil on osteoclastogenesis was blocked by the addition of ODQ and glibenclamide. These results suggest that both the NO and K_{ATP} channel-opening activity of nicorandil inhibit osteoclast differentiation. Further study of nicorandil may lead to the development of drugs for osteoporosis treatment.

1. Introduction

Nicorandil (N-[2-hydroxyethyl]-nicotinamide nitrate) is a hybrid angina therapeutic agent, similar to the nitric acid drugs, which have nitric oxide (NO) action and the ability to open ATP-sensitive K channels (K_{ATP} channels) (Minamiyama et al., 2007). Its pharmacological action involves the activation of guanylate cyclase (GC) by its NO action, followed by increasing cyclic guanosine monophosphate (cGMP) levels. As a consequence, cGMP-dependent protein kinase (PKG) is activated. However, it has the ability to open K_{ATP} channels. This activity increases K^+ flow out of the cell membrane, followed by hyperpolarization and closing of Ca^{2+} channels. As a consequence, the coronary artery relaxes due to the decreasing intracellular Ca^{2+} concentration (Horinaka, 2011; Holzmann et al., 1992).

Bone diseases, such as osteoporosis and periodontal disease, are caused by increased bone resorption resulting from excessive osteoclast activity. Thus, a therapeutic agent that can control the differentiation process is required. Low NO concentrations inhibit osteoclast formation and activity during the osteoclast differentiation stage in murine marrow cultures (Holliday et al., 1997). Furthermore, RANKL, which promotes osteoclast formation, induces expression of nitric oxide synthase (NOS) and NO production in osteoclasts through NF-κB- and IFN-β-mediated mechanisms and regulates excessive cell activity (Zheng et al., 2006). Gene disruption of the entire NOS system enhances BMD and bone turnover in mice in vivo (Sabanai et al., 2008). The endogenous NO / NOS system is important for maintaining bone homeostasis. NO is believed to play a role of suppressing excessive osteoclast formation and bone resorption by self-regulation of osteoclast formation.

Ca^{2+} concentrations in osteoclasts increase upon increases in extracellular ATP (Yu and Ferrier, 1993), which causes the activation of a Ca^{2+}-dependent K^+ channel (Weidema et al., 1997). On the other hand, K_{ATP} channels are inhibited by intracellular ATP concentration increases, and the activation of these channels reduces intracellular Ca^{2+} concentrations. Ca^{2+} functions as a ubiquitous second messenger that can adjust diverse signalling pathways in the differentiation process of osteoclasts (Berridge et al., 2003). Many different stimuli, such as extracellular acidification and Ca^{2+} concentrations, have been shown to regulate the Ca^{2+} concentration in osteoclasts (Xia and Ferrier, 1995; Teti et al., 1989). Calcineurin is a phosphatase that regulates the phosphorylation state of nuclear factor of activated T cell c1 (NFATc1) and is known to be regulated by intracellular Ca^{2+}. Transcription by NFATc1 is greatly influenced by an increase in the intracellular Ca^{2+} concentration (Kim and Kim, 2014; Zhou et al.,

* Corresponding author.
E-mail addresses: g-iwaki@nmc-kobe.org (F. Iwaki), amano@cc.osaka-dent.ac.jp (H. Amano), ohura@cc.osaka-dent.ac.jp (K. Ohura).

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2011).

The objective of this study was to examine the inhibitory effect of nicorandil on the in vitro osteoclast differentiation process and its mechanism.

2. Material and methods

2.1. Cells and materials

Mice were purchased from SHIMIZU Laboratory Supplies Co., Ltd. (Kyoto, Japan). The experiment was conducted according to the implementation guidelines of the Osaka Dental University animal experiment protocol (approval number 15-02009). Human macrophage colony-stimulating factor (M-CSF) and soluble receptor activator of NF-κB ligand (sRANKL) were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was purchased from Cayman Chemicals (Michigan, USA), and glibenclamide was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Nicorandil was a gift from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan).

2.2. Osteoclast formation

According to the method described by Emori et al. (2015), haematopoietic stem cells were collected from 5 to 8-week-old ddY mice in which both ends of the femur and tibia were excised. All bone marrow cells were collected by extrusion with a 27 G micro-injector (Terumo, Tokyo, Japan). The collected cells were suspended in α-minimal essential medium (α-MEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) at pH 7.0, containing 10% foetal bovine serum (FBS; SAFC Biosciences, Inc., Lenexa, KS), 1% GlutaMAX (Invitrogen Corporation, Carlsbad, CA), and Penicillin-Streptomycin Mixed Solution (Nacalai Tesque, Inc., Kyoto, Japan). They were passed through Sephadex G-10 beads (GE Healthcare UK Ltd., Buckinghamshire, UK) packed in disposable Econo-Pac chromatography columns (Bio-Rad, CA, USA). To remove the erythrocytes, the centrifuged cell pellet was re-suspended in 0.83% NH_4Cl-Tris-HCl buffer solution (pH 7.4) and kept on the ice for 20 min. The sample was centrifuged, and the pellet was re-suspended in α-MEM medium. Approximately 5×10^4 cells were seeded into each well of a 96-well plastic culture plate. Each well contained 25 ng/ml M-CSF and 100 ng/ml sRANKL. Five different concentrations of nicorandil (1 μm, 10 μm, 25 μm, 50 μm, and 100 μm) were added to the different experimental groups. Furthermore, two different time-points of nicorandil addition (25 μm, 0–3 days and 4–6 days) were planned. Both the control and experimental groups were cultured at 37 °C in a 5% CO_2/95% air atmosphere for 6 days. The medium was changed every three days.

2.3. Rhodamine phalloidin and tartrate-resistant acid phosphatase (TRAP) staining

After culture, the cells were fixed in 4% paraformaldehyde. The localization of F-actin was observed to confirm differentiation to the active form of osteoclasts. The fixed cells were incubated with 0.1% Triton X for 5 min. Then, the solution was replaced with rhodamine phalloidin solution, and tissues were kept stationary in a dark room for 30 min, followed by F-actin staining. The fluorescence signal was detected by confocal laser scanning microscopy (LSM700, Carl Zeiss, Oberkochen, Germany), and the number of osteoclasts with a fluorescent ring was counted.

For TRAP staining, TRAP solution was added to the well and incubated with the cells at 37 °C for 15 min. The number of cells with 3 or more nuclei was counted under an optical microscope.

2.4. NO production in the culture medium in osteoclast formation assays

NO production was evaluated by measuring NO_2^- and NO_3^- in the culture medium using the Griess method. Using the same procedures and conditions as in the osteoclast formation experiment, cells were incubated with 25 ng/ml M-CSF and 100 ng/ml sRANKL in the presence or absence of 25 μm nicorandil. NO_2^- and NO_3^- in the culture medium supernatants obtained at 0, 1, 3 and 6 days were measured using NO_2/NO_3 Assay Kit-CII (Dojindo Co., Ltd., Kumamoto, Japan).

2.5. Osteo assay

Using the same procedures and conditions used in the osteoclast formation experiment, cells were seeded into Osteo Assay Stripwell Plates (Corning, MA, USA) coated with hydroxyapatite (1×8 wells). In this study, the cells were cultured for 8 days, followed by fixation with 1 M ammonium chloride. For von Kossa staining, 5% silver nitrate solution was added, and the cells were kept stationary for 60 min under light irradiation. Then, 5% sodium thiosulfate was added to develop the black hydroxyapatite. The area of the bone resorption region was quantified with an optical microscope. Quantification was conducted using Adobe Photoshop CC 2014 (Adobe System, CA, USA).

An osteo assay was also performed in the same manner for recovery experiments that examined soluble guanylyl cyclase (sGC) inhibition by ODQ and K_{ATP} channel inhibition by glibenclamide.

2.6. Recovery experiments examining sGC inhibition by ODQ and K_{ATP} channel inhibition by glibenclamide

To verify the pharmacological mechanisms of osteoclast inhibition by nicorandil, we performed an experiment in which sGC and K_{ATP} channel opening were blocked by the addition of ODQ and glibenclamide during the osteoclast formation process (Marinko et al., 2015). ODQ was dissolved in dimethyl sulfoxide (DMSO), and glibenclamide was dissolved in N-N-dimethylformamide (DMF).

Cells were seeded onto 96-well plastic culture plates (5×10^4 cells/well) with α-MEM medium (containing 10% FBS, 25 ng/ml M-CSF, and 100 ng/ml sRANKL). Because the IC_{50} of nicorandil was 25 μm in the osteoclast formation experiment, a concentration of 25 μm was chosen. Four groups were investigated: 10 μm ODQ+25 μm nicorandil, 10 μm glibenclamide+25 μm nicorandil, 10 μm ODQ+10 μm glibenclamide+25 μm nicorandil, and 25 μm nicorandil alone (control group). Furthermore, to verify the effects of authentic NO production, an experiment was performed by adding 10 μm ODQ in osteoclast formation assay. Cells were cultured at 37 °C in a 5% CO_2/95% air atmosphere for 6 days. The medium was changed every three days.

2.7. Statistical analysis

The data are shown as the means ± standard error of mean. Statistical significance was defined at a value of $P < 0.05$. Analysis of statistically significant differences between multiple groups was conducted using one-way analysis of variance with the statistical software SPSS, version 16 (IBM Japan, Tokyo, Japan) with a Bonferroni correction.

3. Results

3.1. Nicorandil induces NO production in osteoclastogenesis

NO production increased in a time-dependent manner only in the presence of nicorandil (Fig. 1A).

The number of osteoclasts with actin rings was 84.83 ± 4.57 in the early time-point (0–3 days) nicorandil group and 59.33 ± 4.09 in the