European Journal of Pharmacology 793 (2016) 14–20

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Endocrine pharmacology

Nicorandil inhibits osteoclast differentiation in vitro

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ARTICLE INFO

Keywords:
Nicorandil
Suppression of osteoclast differentiation
Osteoporosis

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.,

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http://dx.doi.org/10.1016/j.ejphar.2016.10.034
Received 12 May 2016; Received in revised form 25 October 2016; Accepted 26 October 2016
Available online 01 November 2016
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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

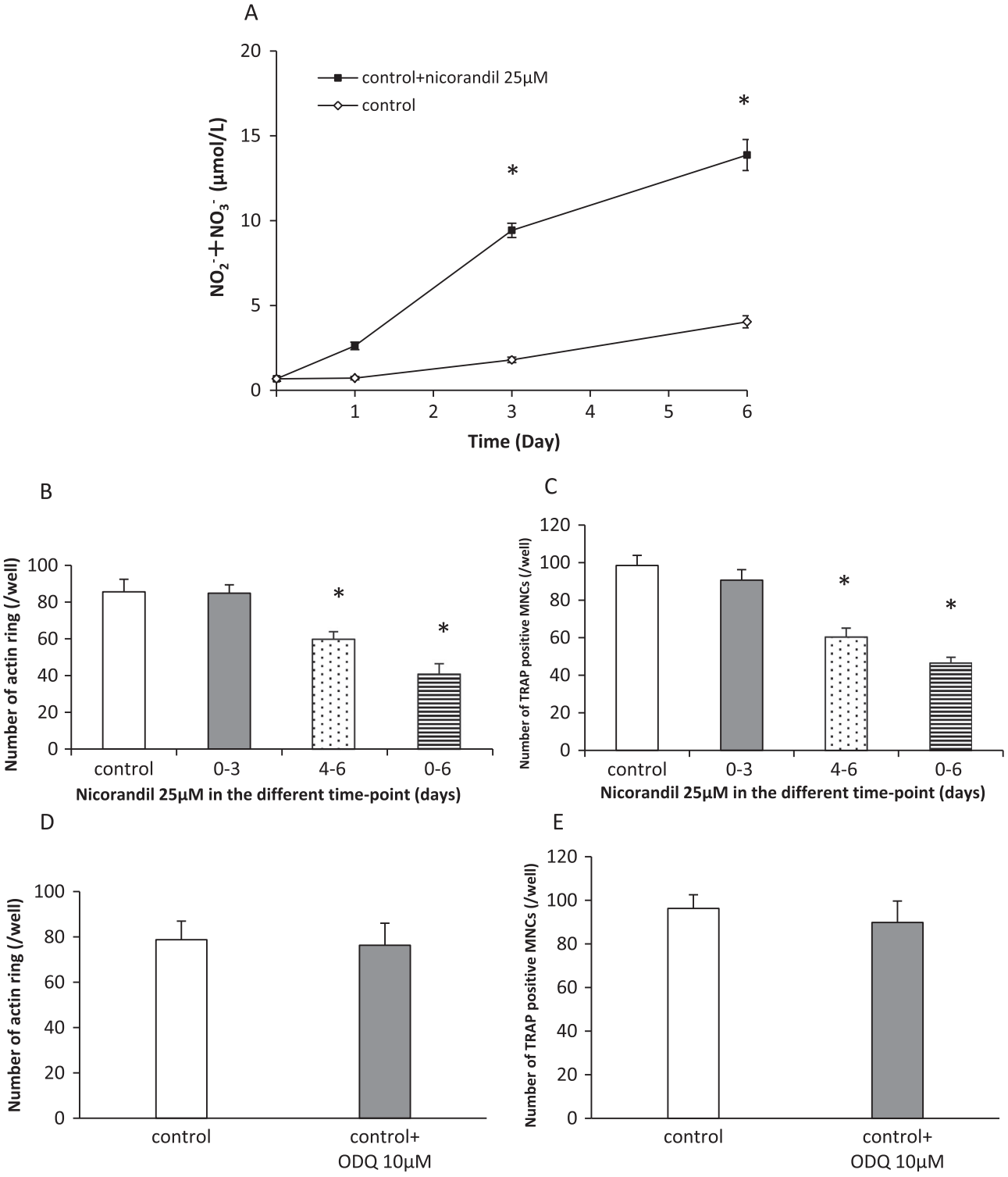

Fig. 1. Nicorandil induces NO production in osteoclastogenesis. NO production increased in a time-dependent manner only in the presence of nicorandil (A). The number of osteoclasts with actin rings decreased markedly in the late time-point group compared with the number observed in the early time-point group (B). After TRAP staining, the number of multinuclear cells decreased markedly in the late time-point group (4–6 days) compared with the number in the early time-point group (0–3 days) (C). The number of osteoclasts was not increased by inhibiting sGC using ODQ in the osteoclast formation assay (D and E). *P < 0.01 vs. control group.

late time-point (4–6 days) nicorandil group. The number of osteoclasts with actin rings decreased markedly in the late time-point group compared with the number observed in the early time-point group (Fig. 1B).

After TRAP staining, the number of osteoclasts was 90.67 ± 5.61 in the early nicorandil time-point group and 59.88 ± 4.70 in the late nicorandil time-point group. The number of multinuclear cells decreased markedly in the late time-point group (4–6 days) compared with the number in the early time-point group (0–3 days) (Fig. 1C).

The number of osteoclasts was not increased by inhibiting sGC using ODQ in the osteoclast formation assay (Fig. 1D and E).

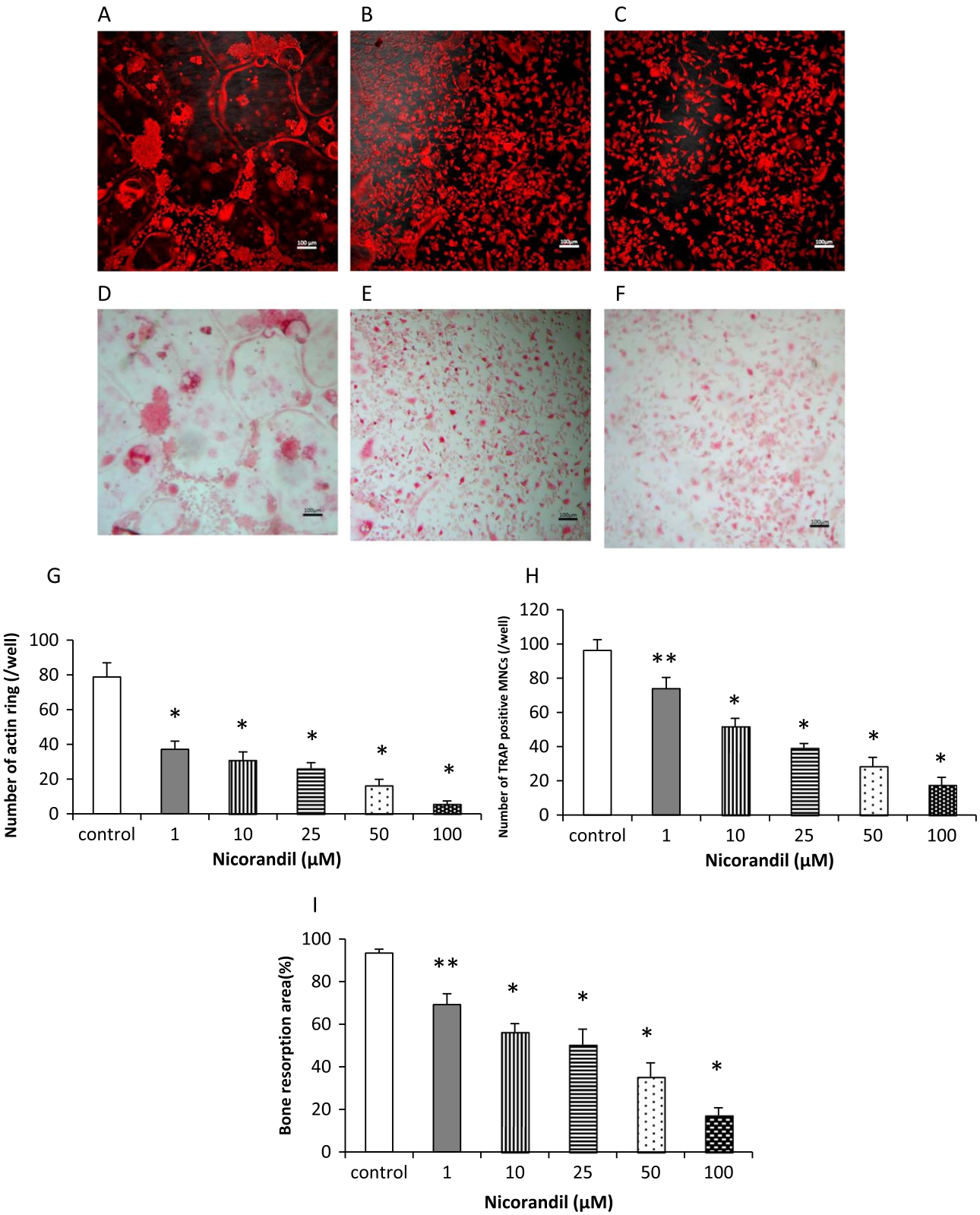

Fig. 2. Effects of nicorandil on osteoclast formation and differentiation. A and D; control group (25 ng/ml M-CSF and 100 ng/ml sRANKL). B and E; nicorandil 10 μm group. C and F; nicorandil 50 μm group. Observation of the fluorescence signal after rhodamine phalloidin staining revealed that active osteoclasts with F-actin rings no longer increased in size, and the number of cells also decreased markedly with nicorandil in a concentration-dependent manner (A–C and G). After TRAP staining, osteoclast formation was observed in the control group. In the nicorandil groups, the size and number of osteoclasts decreased in a concentration-dependent manner (D–F and H). After von Kossa staining, the apatite resorption area was significantly decrease in a concentration-dependent manner (I). **P < 0.05 vs. control group, *P < 0.01 vs. control group.

3.2. Nicorandil inhibits osteoclast formation and differentiation

Observation of the fluorescence signal after rhodamine phalloidin staining revealed that active osteoclasts with F-actin rings no longer increased in size, and the number of cells also decreased markedly with nicorandil in a concentration-dependent manner (Fig. 2A–C). After

TRAP staining, osteoclast formation was observed in the control group. In the nicorandil groups, the size and number of osteoclasts decreased in a concentration-dependent manner (Fig. 2D–F). The number of osteoclasts with actin rings was 78.75 ± 8.79 in the control group and 37.13 ± 5.01 in the group treated with 1 μm nicorandil, half the number of the control group. The number of osteoclasts with actin rings was reduced gradually in a concentration-dependent manner within the range of 10–100 μm (Fig. 2G). After TRAP staining, the number of osteoclasts was 96.25 ± 6.57 in the control group, 51.75 ± 4.76 in the group treated with 10 μm of nicorandil and 39.08 ± 2.73 in the group treated with 25 μm of nicorandil. The IC_{50} was between 10 and 25 μm (Fig. 2H). Although inhibition of differentiation of osteoclasts was statistically significant, numerous undifferentiated cells were still present, and no significant cytotoxicity was observed, even at a concentration of 100 μm.

After von Kossa staining, the bone resorption rate was 93.40 ± 1.94% in the control group, indicating that the hydroxyapatite was almost completely absorbed. However, the bone resorption rate was 56.36 ± 4.27% in the 10 μm nicorandil group and 35.3 ± 6.98% in the 50 μm nicorandil group. This decrease in the area of apatite resorption was dose-dependent and statistically significant (Fig. 2I).

3.3. Recovery experiments examining sGC inhibition by ODQ and KATP channel inhibition by glibenclamide

To analyse the number of TRAP-positive cells as an index, the group treated with 25 μm of nicorandil (approximately the IC_{50}) was used as the control group. After both rhodamine phalloidin and TRAP staining, the more osteoclasts were present in the group treated with glibenclamide and the group treated with ODQ. The difference was even larger in the group treated by both agents (Fig. 3A–F).

The number of osteoclasts with actin rings was 21.33 ± 3.76 in the control group and two-fold higher (37.67 ± 2.81) in the group treated with 10 μm glibenclamide. The number was increased to 40.00 ± 4.97 in the group treated with 10 μm ODQ and significantly increased in the group treated with 10 μm glibenclamide+10 μm ODQ (72.67 ± 19.82) (Fig. 3G). After TRAP staining, the number of osteoclasts was 18.50 ± 4.18 in the control group. The number was increased to 34.83 ± 5.72 in the group treated with 10 μm glibenclamide and 37.83 ± 6.33 in the group treated with 10 μm ODQ. In the group treated with 10 μm glibenclamide and 10 μm ODQ, the number was significantly increased to 69.83 ± 18.29, which was equivalent to that of the group not treated with nicorandil (Fig. 3H).

After von Kossa staining, the bone resorption rate was 50.26 ± 6.33% in the control group. The rate was 88.18 ± 3.87% in the 10 μm glibenclamide group and 97.78 ± 0.34% in the 10 μm ODQ group. In the 10 μm glibenclamide+10 μm ODQ group, the bone resorption rate was 99.12 ± 0.37%, and the apatite resorption area was significantly increased (Fig. 3I).

4. Discussion

Nicorandil is a hybrid agent for angina therapy that has NO action and the ability to open K_{ATP} channels. The pharmacological action of NO in the myocardium is evident during the activation of cGMP-dependent PKG via activation of GC and the resulting increase in cGMP levels. NO synthesis involves NOS, and it has been implicated in blood pressure adjustment and apoptosis as a cell signalling factor.

NOS proteins are classified as constitutive NOSs (cNOSs), which always exist intracellularly at certain levels, and inducible NOSs (iNOSs), which are induced by inflammation and stress. MacIntyre et al. (1991) showed experimentally that NO inhibits the activity of isolated rat osteoclasts and decreases the bone resorption area. Thereafter, Kasten et al. (1994) showed enhanced bone resorption in birds and mammals both in vitro and in vivo after inhibition of NOS, as well as direct involvement of NO in osteoclast activity. Furthermore, Zheng et al. (2006) demonstrated that RANKL induces iNOS expression and NO production in osteoclasts via NF-κB- and IFN-β-mediated mechanisms and regulates excessive cellular activity. Endogenous NO produced by iNOS has been reported to affect bone remodelling during fracture healing and in osteoarthritis (McCartney-Francis et al., 2001; Diwan et al., 2000). Gene disruption of the entire NOS system enhances BMD and bone turnover in mice in vivo (Sabanai et al., 2008). According the above reports, NO is believed to play a role in suppressing excessive osteoclast formation and bone resorption by self-regulation of osteoclast formation.

NO is the primary activator of sGC and increases cGMP levels (Derbyshire and Marletta, 2012). Various concentrations of NO regulate osteoclast motility via cGMP-dependent protein kinase I and vasodilator-stimulated phosphoprotein (Yaroslavskiy et al., 2005). Homer et al. (2015) demonstrated that administration of sGC agonists induces bone resorption, remodelling, and new bone formation in vivo.

In the present experiment, little authentic NO production was detected in the culture medium in osteoclast formation assays. Osteoclast inhibition by authentic NO production was not observed in vitro. NO production was increased only in the presence of nicorandil. The number of osteoclasts decreased markedly in the late time-point nicorandil-addition group compared with the number in the early time-point nicorandil-addition group. The number of osteoclasts and the bone resorption area were increased by inhibiting sGC by adding ODQ to 25 μm of nicorandil.

These results suggested that increased cGMP levels caused by the activation of GC interacted with osteoclasts and sent a signal to inhibit the differentiation of mononuclear osteoclasts into mature multinuclear osteoclasts in accordance with increased NO concentrations.

The K_{ATP} channel was first discovered in cardiomyocytes by Noma (1983). Subsequent pharmacological studies revealed that the channel exists not only in cardiomyocytes but also in pancreatic β cells, vascular smooth muscle cells, and nerve cells (Ashcroft, 1988). When the K_{ATP} of vascular smooth muscle cells is activated and the cell membrane is depolarized, voltage-dependent Ca^{2+} channels are closed, the intracellular Ca^{2+} concentration decreases, and the smooth muscle relaxes (Quayle et al., 1997). Ca^{2+} concentrations in osteoclast increase with increasing extracellular ATP (Yu and Ferrier, 1993), which leads to activation of a Ca^{2+}-dependent K^+ channel (Weidema et al., 1997). However, K_{ATP} channels are inhibited by increased intracellular ATP concentrations, and the activation of these channels reduces intracellular Ca^{2+} concentrations.

Intracellular Ca^{2+} kinetics are deeply involved in the differentiation and activation of osteoclasts (Fu et al., 2015; Hwang and Putney, 2011; Amano et al., 2000). In the differentiation process of osteoclasts, RANKL produced by osteoblasts binds to RANK, and molecules such as TNF receptor-associated factor 6 (TRAF6) assemble at the intracellular domain of RANK. Then, kinases, such as extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, are activated. Finally, cell differentiation is proceeded by transcriptional regulation via transcription factors, such as c-Fos, NFATc1, and PU.1 (Boyle et al., 2003; Teitelbaum, 2000). The expression level of the NFATc1 gene is dramatically increased by RANK signalling during the early stages of osteoclast differentiation. This activation requires the activation of Ca^{2+}-dependent signalling, and RANK signalling also regulates the activation of this pathway (Takayanagi et al., 2002). However, NFATc1-mediated transcription is not fully activated by only a transient increase in Ca^{2+}, but rather a sustained elevated level of Ca^{2+} is required. To maintain the increase in intracellular Ca^{2+} levels, Ca^{2+} influx from outside the cell is required, and this mechanism is referred to as store-operated Ca^{2+} entry (SOCE) (Parekh and Putney, 2005). SOCE promotes Ca^{2+} influx from the extracellular space through Ca^{2+} channels on the plasma membrane by sensing the depletion of Ca^{2+} in the endoplasmic reticulum. This phenomenon, which is dependent on the Ca^{2+} concentration in the endoplasmic reticulum, is called capacitative Ca^{2+} entry (CCE) and is known to be the

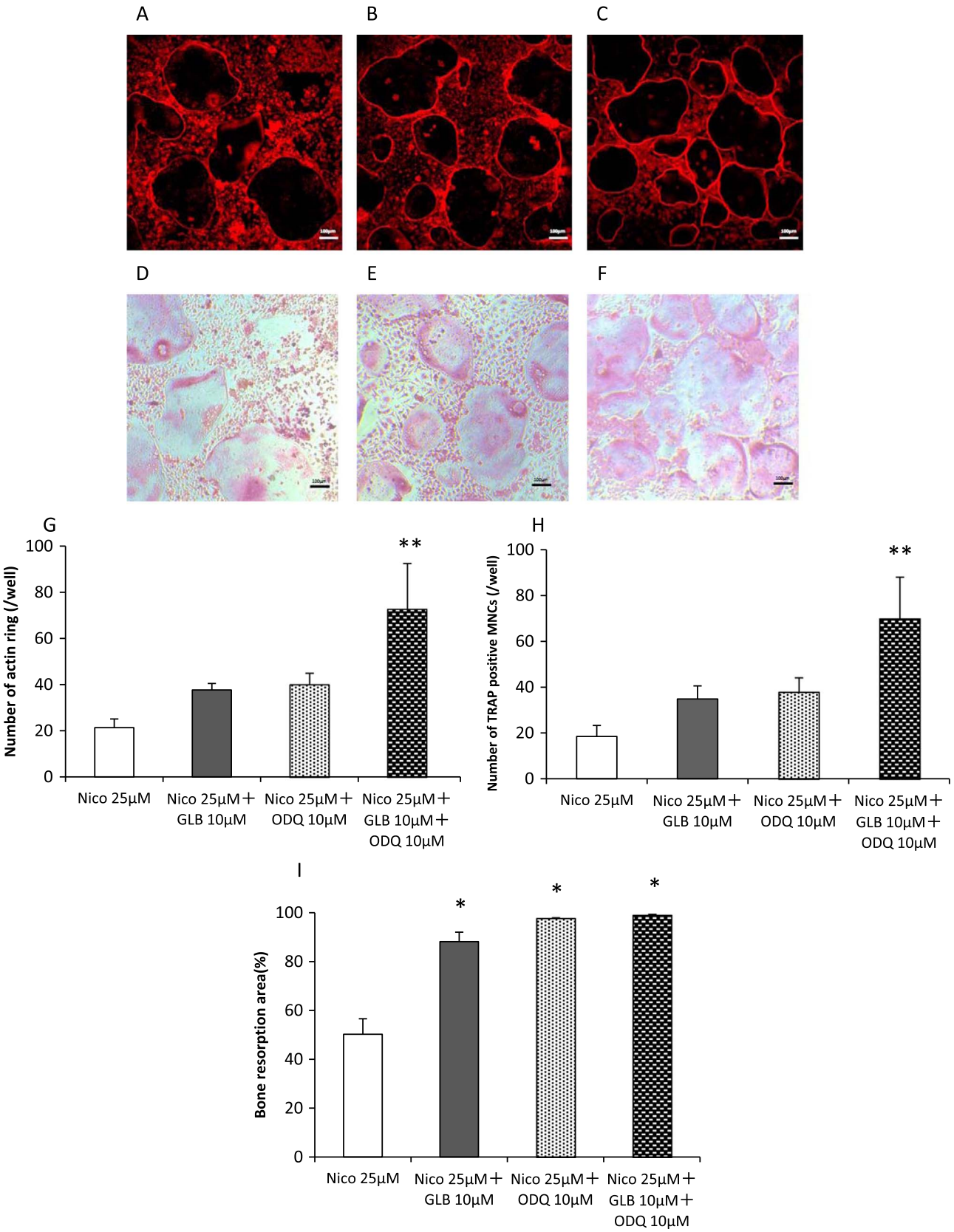

Fig. 3. Study of the mechanism of nicorandil action using various inhibitors. A and D; 25 μm nicorandil +10 μm glibenclamide group, B and E; 25 μm nicorandil +10 μm ODQ group, C and F; 25 μm nicorandil +10 μm glibenclamide +10 μm ODQ group. After both rhodamine phalloidin and TRAP staining, the more osteoclasts were present in the group treated with glibenclamide and with ODQ. The difference was even larger in the group treated by both agents (A–F). The number of osteoclasts with actin rings was increased in the group treated with 10 μm glibenclamide and with 10 μm ODQ. The number of osteoclasts with actin rings was significantly increased in the group treated by both agents (G). After TRAP staining, the number of osteoclasts was increased in the group treated with 10 μm glibenclamide and with 10 μm ODQ. The number of osteoclasts was significantly increased in the group treated by both agents (H). After von Kossa staining, the apatite resorption area was significantly increase in the group treated with 10 μm glibenclamide and with 10 μm ODQ (I). Nico, nicorandil; GLB, glibenclamide. **P < 0.05 vs. nicorandil 25 μm group, *P < 0.01 vs. nicorandil 25 μm group.

mechanism responsible for the sustained Ca^{2+} signal. In other cells of the haematopoietic system, SOCE is known to play an important role in the activation of NFAT signalling (Oh-Hora, 2009; Feske et al., 2003).

In this experiment, osteoclast formation was restored by addition of glibenclamide, which inhibited the K_{ATP} channel-opening activity of nicorandil. This finding demonstrated that K_{ATP} channel-opening activity might result in the suppression of osteoclast differentiation. This result suggested the following mechanism of nicorandil action. It inhibits SOCE by depolarization, as seen in vascular smooth muscle. As a consequence, the Ca^{2+} concentration in osteoclast precursor cells decreases. Thus, cell differentiation by NFATc1 is inhibited.

In conclusion, our results suggested that through its NO action and ability to open K_{ATP} channels, nicorandil inhibits the differentiation and activity of osteoclasts in vitro. Further study of nicorandil might lead to the development of osteoporosis treatment drugs.

Acknowledgements

This study was supported by the Ministry of Education, Culture, Sports, Science and Scientific Research Basic Research C (No. 25462899). We thank Drs. Junichi Kikuta M.D., Ph.D., Keita Aoi M.D. and Prof. Masaru Ishii M.D., Ph.D. for instruction on confocal laser microscope imaging and Drs Hiroshi Kajiya Ph.D., Koji Okabe D.D.S. Ph.D. and Yoichi Miyamoto Ph.D. for helpful discussion. We also thank Chugai Pharmaceutical Co., Ltd., for providing nicorandil.

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