Nicorandil Inhibits Angiotensin II-induced Proliferation of Cultured Rat Cardiac Fibroblasts

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Abstract

Background/Aims: Nicorandil, a K_{ATP} channel opener, nitric oxide donor and antioxidant, was shown to exert a variety of pharmacological effects including cardioprotective properties. However, its mechanisms of the action are not completely understood. The aims of this study were to examine whether nicorandil may alter angiotensin II (Ang II)-induced cell proliferation and to identify the putative underlying signaling pathways in rat cardiac fibroblasts. Methods: Cultured rat cardiac fibroblasts were pretreated with nicorandil then stimulated with Ang II, cell proliferation and endothelin-1 (ET-1) expression was examined. The effect of nicorandil on Ang II-induced reactive oxygen species (ROS) formation; and extracellular signal-regulated kinase (ERK) phosphorylation were also examined. In addition, the effect of nicorandil on nitric oxide (NO) production, and endothelial nitric oxide synthase (eNOS) phosphorylation were tested to elucidate the intracellular mechanism. Results: Nicorandil (0.1-10 μM) caused a concentration-dependent inhibition of Ang II-increased cell proliferation and ET-1 expression which were prevented by the K_{ATP} channel blocker-glibenclamide (1μM). Nicorandil also inhibited Ang II-increased ROS formation, and ERK phosphorylation. In addition, nicorandil was found to increase the NO generation, and eNOS phosphorylation. N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS, and the short interfering RNA transfection for eNOS markedly attenuated the inhibitory effect of nicorandil on Ang II-induced cell proliferation. Conclusion: Our results suggest that nicorandil prevents cardiac fibroblast proliferation by interfering with the generation of ROS and involves the activation of the eNOS-NO pathway.

Keywords: Nicorandil; Angiotensin II; Cardiac Fibroblast Proliferation; Nitric Oxide.

Introduction

Nicorandil, a hybrid antianginal drug that combines the characteristics of an ATP-sensitive potassium (K_{ATP}) channel opener and a nitric oxide (NO) donor, exerts beneficial effects on myocardial performance [1]. The K_{ATP} channel plays an important role not only in coronary blood flow regulation, but also in the protection of cardiomyocytes against ischemia–reperfusion injury [2-4]. However, the pretreatment effects and mechanisms of nicorandil on cardio-protections are not well understood. Cardiac fibrosis, characterized by the proliferation of cardiac fibroblasts and abundant accumulation of matrix proteins in the extracellular space, is one of the deleterious events accompanying hypertension that may participate in the progression toward heart failure [5]. Angiotensin II (Ang II), the effector peptide of the renin-angiotensin system, is a key pathogenic factor in the development of heart failure. Ang II induces cardiac myocyte hypertrophy, fibroblast proliferation, and collagen formation [5]. It is well known that Ang II stimulates reactive oxygen species (ROS) in cardiovascular tissues, which is involved in cardiac remodeling [6]. We have reported that ROS are essential for Ang II-induced endothelin-1 (ET-1) gene expression and cell proliferation in cardiac fibroblasts [7]. Nicorandil is known to act as an antioxidant, reacting with free radicals to prevent oxidative cell damage in several types of preparations [8]. Application of nicorandil was also reported to stimulate the production of NO from cultured vascular endothelial cells [9]. In addition, Horinaka *et al* have demonstrated that nicorandil increases cardiac endothelial NO synthase (eNOS) expression by activating K_{ATP} channels in animal studies [10-12]. NO derived from eNOS was reported to play a key role in the regulation of cardiac

fibrosis [13]. However, no study has addressed the effects of nicorandil on cardiac fibroblast proliferation. The aims of this study were to investigate the anti-proliferative effect of nicorandil on Ang II-induced cardiac fibroblast proliferation and to identify whether the underlying mechanisms are associated with the NO-dependent pathway.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and tissue culture reagents were from Life Technologies, Inc (Gaithersburg, MD. U.S.A). 2',7'-Dichlorofluorescin diacetate (DCF-DA) was obtained from Molecular Probes (Eugene, OR, U.S.A.). Nicorandil was kindly provided by Chugai (Tokyo, Japan) and was dissolved in dimethyl sulfoxide (DMSO), and the DMSO content in all groups was 0.1%. N-nitro-L-arginine methyl ester (L-NAME) and all other reagent-grade chemicals were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.). Rabbit polyclonal anti-phospho-ERK antibody and anti-ERK antibody were purchased from New England Biolabs (Beverly, MA, U.S.A.) and Santa Cruz Biotechnology Inc (Santa Cruz, CA, U.S.A.). Anti-Ser1177 phospho-eNOS antibodies were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.); and anti-eNOS antibodies were from BD Bioscience (San Jose, CA, U.S.A).

Culture of Cardiac Fibroblasts

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was also approved by the Institutional Animal Care and Use Committee of China Medical University. Primary cultures of neonatal rat cardiac fibroblasts were prepared as previously described [7]. Briefly, ventricles from 1- to 2-day-old neonatal Sprague-Dawley rats were cut into chunks of approximately 1 mm^3 by using scissors and were subjected to trypsin (0.125%; Invitrogen, Carlsbad, CA, U.S.A.) digestion in phosphate

buffered saline (PBS). Dispersed cells were incubated on 100-mm culture dishes for 30 min in a 5% CO_2 incubator. Nonmyocytes attached to the bottom of the dishes were subsequently incubated with DMEM supplemented with 10% fetal calf serum for an additional 2 to 4 d. Confluent nonmyocytes were treated with trypsin and subcultured. Subconfluent (~70% confluency) cardiac fibroblasts grown in culture dishes from the second to fourth passage were used in the experiments and were > 99% positive for vimentin antibodies (Sigma-Aldrich). Serum-containing medium from the cultured cells was replaced with serum-free medium, and the cells were then exposed to the agents as indicated.

Cell Proliferation

Proliferation was assessed by quantifying 5-bromo-2'-deoxyuridine (BrdU) incorporation in the presence or absence of reagents as indicated. The rate of cellular proliferation was determined by cell counting. Cells were removed from the culture dish by addition of trypsin and then centrifuged. The pellet was resuspended in 1 ml DMEM and cells were counted in an automatic cell counter (S.ST.II/ZM, Coulter Electronics Ltd., Miami, FL, U.S.A.). Cell proliferation was assessed by the incorporation of BrdU. Cells (1×10^4 cells/well) were incubated in 96-well plastic plates. Then, BrdU (10 μM) was added to the medium and the cells were incubated for another 18 h. Subsequently, the cells were fixed and BrdU incorporation was determined with a Cell Proliferation enzyme-linked immunosorbent assay (ELISA) Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

RNA Extraction and Quantitative Polymerase Chain Reaction (Q-PCR) Analysis

Total RNA was extracted from cardiac fibroblasts using the TRIzol method according to the protocol recommended by the manufacturer (Invitrogen, Carlsbad, CA, USA), and used to synthesize single-stranded complementary (c)DNA with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). ET-1 mRNA was quantified using TaqMan Gene Expression Master Mix (Applied Biosystems) with specific primers in an ABI 7300 Real-Time PCR System (Applied Biosystems). TaqMan Gene Expression Assay kits containing specific primers for ET-1 (lot no. Rn00561129_m1), and GAPDH (lot no. Rn01775763_g1) were obtained from Applied Biosystems. The specific primers for GAPDH were used to normalize the amount of sample added. The relative quantitation of ET-1 mRNA was analyzed using the comparative Ct method. All quantifications were performed in triplicate samples for three separate experiments.

Measurement of ET-1 Concentration

ET-1 levels were measured in culture medium using a commercial enzyme-linked immunosorbent assay kit (Amersham-Pharmacia). Results were normalized to cellular protein content in all experiments, and expressed as a percentage relative to the control group.

Flow Cytometric Assay of 2',7'-dichlorodihydrofluorescein Oxidation

The determination of intracellular reactive oxygen species production was based on the oxidation of 2′,7′-dichlorodihydrofluorescein (DCFH) to a fluorescent 2′,7′-dichlorofluorescein (DCF). DCFH was added at a final concentration of 10 μM and incubated for 30 min at 37°C. The cells were then washed once with PBS and maintained in a 1-ml culture medium. Following drug treatment, the medium was aspirated and cells were washed twice with PBS, and then dissociated with trypsin. Cellular fluorescence was determined by flow cytometry (FACS-SCAN, Becton-Dickinson, Franklin Lakes, NJ, U.S.A.). Cells were excited with an argon laser at 488 nM, and measurements were taken at 510–540 nm.

Western Blot Analysis

Western blot analysis was performed as previously described [7]. Whole-cell extracts were obtained in a RIPA buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, 1.0% Triton X-100, 1% Deoxycholate, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, and protease inhibitor cocktail; Complete, Roche Diagnostics GmbH, Germany). Extracts or proteins were separated by SDS-PAGE followed by electrotransfer to polyvinylidene difluoride (PVDF) membranes and probed with antisera, followed by horseradish peroxidase-conjugated secondary antibodies. The proteins were visualized by chemiluminescence, according to the manufacturer's instructions (Pierce Biotechnology Inc., IL, U.S.A.).

Measurement of Nitrate/Nitrite Levels

The culture medium was stored at –70 ºC until use. After the medium had been

thawed, the sample was deproteined with two volumes of 4^{o}C 99% ethanol and centrifuged (3000g for 10 min). These medium samples (100 µl) were injected into a collection chamber containing 5 % VCl_3. This strong reducing environment converts both nitrate and nitrite to nitric oxide. A constant stream of helium gas carried nitric oxide into a nitric oxide analyzer (Seivers 270B NOA; Seivers Instruments Inc., Boulder, CO, U.S.A.), where nitric oxide reacted with ozone, resulting in the emission of light. Light emission is proportional to the quantity of nitric oxide formed; standard amounts of nitrate were used for calibration.

Short Interfering RNA (siRNA) Transfection

We purchased eNOS siRNA from Santa Cruz Biotechnology (CA, U.S.A.). eNOS siRNA, and mock control oligonucleotides were transfected using the Lipofectamine reagent according to the manufacturer's instructions. The final concentration of siRNAs for transfection was 100 nM. We washed transfected cells and incubated them in new culture media for an additional treatment as indicated.

Statistical Analysis

Results are expressed as mean $\pm$ S.E.M. for at least six experiments unless designated otherwise. Statistical analysis was performed using Student's t test or analysis of variance (ANOVA) followed by Tukey's multiple comparisons using GraphPad Prism (GraphPad Software, San Diego, CA, U.S.A). A value of $P < 0.05$ was considered to be statistically significant.

Results

Effects of Nicorandil on Ang II-induced Cell Proliferation of Cardiac Fibroblasts

Exposure of cultured cardiac fibroblasts to nicorandil (0.1, 0.3, 1, 3, 10, or 30 μM) for 24 h did not induce any significant effect on cardiac fibroblasts viability (data not shown). The effects of nicorandil on Ang II-stimulated rat cardiac fibroblast proliferation were assessed by analyzing DNA synthesis with BrdU incorporation and cell counting. Pretreatment of cardiac fibroblasts with nicorandil (1-10 μM) for 30 min followed by exposure to Ang II (100 nM) for 24 h, resulted in a significant decrease in Ang II-increased cell number and BrdU incorporation (Figure 1A, 1B). The involvement of the K_{ATP} channel in the effects of nicorandil on fibroblast proliferation was also confirmed by experiments performed in the presence of 1 μM glibenclamide. In the presence of glibenclamide, 10 μM nicorandil failed to inhibit Ang II-increased cell number and BrdU incorporation. These data clearly suggest that nicorandil inhibited Ang II-induced proliferation of cardiac fibroblasts and confirm the role of the K_{ATP} channel in the mechanisms of action of the cardiovascular effects of nicorandil.

Effects of Nicorandil on Ang II-induced ET-1 Expression in Cardiac Fibroblasts

Cardiac fibroblasts were preincubated with nicorandil (0.1, 0.3, 1, 3, and 10 μM, 30 min) before exposure to Ang II (100 nM) for 30 min, and then assayed for nicorandil-inhibited Ang II-induced ET-1 expression. Nicorandil (1-10 μM) caused a down-regulation of Ang II-induced ET-1 mRNA (Figure 2A). Exposure of cardiac fibroblasts to Ang II (100 nM) for 24 h significantly increased ET-1 peptide secretion (Figure 2B). Pretreatment of cardiac fibroblasts with

nicorandil (1-10 μM) inhibited Ang II-induced ET-1 secretion (Figure 2B). In the presence of glibenclamide, 10 μM nicorandil failed to inhibit Ang II-induced ET-1 secretion. These data indicate that nicorandil inhibited Ang II-induced ET-1 expression in cardiac fibroblasts.

Effects of Nicorandil on Ang II-increased ROS Formation and ERK Phosphorylation

We previously reported that ROS were involved in the activation of the ERK pathway, which culminated in ET-1 gene expression [7]. In this study, we further examined whether nicorandil prevented Ang II-increased ROS formation and ERK phosphorylation in cardiac fibroblasts. As shown in Figure 3A, nicorandil (3 μM) or the ROS scavenger *N*-acetylcysteine (NAC; 5 mM) pretreatment significantly inhibited Ang II-induced ROS formation. As shown in Figure 3B, exposure of cardiac fibroblasts to Ang II (100 nM) for 30 min rapidly activated phosphorylation of ERK. However, cardiac fibroblasts pretreated with nicorandil (3 μM, 30 min) or NAC (5 mM) had significantly decreased levels of Ang II-induced ERK phosphorylation. These findings support that nicorandil inhibited Ang II-increased intracellular ROS levels and ERK signaling pathway in cardiac fibroblasts.

Effects of Nicorandil on Nitric Oxide Synthesis and Phospho-eNOS in Cardiac Fibroblasts

Exposure of cardiac fibroblasts to nicorandil time-dependently enhanced nitric oxide generation (Figure 4A). In addition, nicorandil treatment in cardiac fibroblasts also significantly enhanced phospho-eNOS (Figure 4B). These

findings reveal that nicorandil increased nitric oxide production and eNOS phosphorylation in cardiac fibroblasts.

Role of eNOS-NO Pathway in the Inhibitory Effect of Nicorandil on Ang II–induced Cardiac Fibroblast Proliferation.

To identify the signaling pathways involved in the effect of nicorandil, a NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME) and the short interfering RNA transfection for eNOS were applied in cardiac fibroblasts. The inhibitory effect of nicorandil on the Ang II-increased BrdU incorporation and cell proliferation were reduced by L-NAME or the short interfering RNA transfection for eNOS (Figure 5A, 5B). These results reveal the involvement of eNOS-NO signaling pathway in nicorandil's effect on Ang II-induced cardiac fibroblast proliferation.

Discussion

The major finding of this study is that nicorandil inhibits cardiac fibroblast proliferation by interfering with the generation of ROS and involves the activation of the eNOS-NO pathway.

The increased levels of different humoral factors such as Ang II and ET-1 may cause the development of cardiac hypertrophy and cardiac fibrosis, leading to an enhancement of cardiac remodeling during the development of heart failure [14, 15]. Cardiac fibrosis is a characterization of heart disease and is the result of a variety of structural changes that occur after pathological stimuli to the cardiovascular system [15]. Fibroblasts play a pivotal role in the development of cardiac fibrosis, progression of left ventricular remodeling and results in diastolic dysfunction, accounting for 50% to 70% of congestive heart failure in clinical practice [15]. Experimental studies have shown cardioprotective effeects of nicorandil; however, there is no existing study addressing the interference of nicorandil on ET-1 expression, ROS and NO production in cardiac fibroblasts. The results from our study demonstrate that nicorandil inhibited Ang II-induced ET-1 expression and cell proliferation of cardiac fibroblasts. It is considered that ROS are mediators for intracellular signaling, which may involve the induction and/or the development of various physiological and pathophysiological events such as proliferation of cardiac fibroblasts [7]. In addition, elevated ROS levels are involved in cell proliferation and ET-1 induction which can be attenuated by antioxidant pretreatment of cells [7]. More specifically, nicorandil also prevented Ang II-increase ROS generation (Figure 3A). In particular, including our previous report has been demonstrated that activation of ERK is redox-sensitive and that suppression of

ROS generation, inhibits ET-1 expression may have benefit effect of cell-protection [7]. To gain insight into the mechanism of action of nicorandil, we examined whether nicorandil affected Ang II-activated ERK pathway of cardiac fibroblasts. As shown in Figure 3B, exposure of cardiac fibroblasts to Ang II (100 nM) for 30 min rapidly activated phosphorylation of ERK. However, cardiac fibroblasts pretreated with nicorandil (3 μM, 30 min) had significantly decreased levels of Ang II-induced ERK phosphorylation. Studies on the anti-oxidative effect of nicorandil in inhibiting radical production revealed that it possesses a certain degree of protective effects *in vitro* and *in vivo* [9, 16-18]. The protective characteristics may be associated with their antioxidant activity.

It has been reported that NO can trigger apoptosis or protect cells from apoptotic stimuli, depending on the concentration of NO [19]. In addition, NO has also been suggested to inhibit fibroblasts proliferation [20, 21]. Nicorandil can stimulate NO release from cultured human umbilical vein endothelial cells [9]. In this study; we clearly demonstrated that nicorandil treatment in cardiac fibroblasts significantly enhanced phospho-eNOS. Our findings reveal that nicorandil increased NO production and the eNOS-NO signaling pathway was involved in nicorandil's effect on Ang II-induced cardiac fibroblast proliferation.

In summary, this study has shown that nicorandil inhibited Ang II-induced ROS formation, ERK phosphorylation, ET-1 expression, and cell proliferation. Moreover, nicorandil also increased eNOS phosphorylation, and thereafter NO production in cardiac fibroblasts. It appears plausible that the Ang II-activated signaling pathway consists of a number of redox-sensitive steps and that nicorandil treatment could modulate the redox state of the cell. The present study delivers important new insight into the molecular mechanisms of action

of nicorandil in cardiac fibroblasts.

Acknowledgments

This work was supported with grants from National Science Council Grant (NSC97-2320-B-038-008-MY3), Shin Kong Wu Ho-Su Memorial Hospital (SKH-TMU-93-03 to J-YL and J-CL) and China Medical University (CMU-98-S-22 to T-HC).

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Figure Legends

Figure 1. Effects of nicorandil on the Ang II-induced cell proliferation in cardiac fibroblasts. Cells were preincubated with the indicated concentrations of nicorandil and treated with Ang II (100 nM) for 24 h as indicated. Glibenclamide (1 μM), a K_{ATP} channel blocker, was applied in some groups. BrdU incorporation and cell number were expressed as percentage of control (Ctrl). Results were shown as mean ± S.E.M. (n=6). *P<0.05 versus control; # P<0.05 versus Ang II alone; +P<0.05 versus the nicorandil and Ang II treatment.

(A) Nicorandil inhibits Ang II-induced DNA synthesis. Cell proliferation was estimated from the incorporation of BrdU and calculated as a percentage of the control value.

(B) Nicorandil inhibits Ang II-induced cell proliferation. Cells were counted for cell number and calculated as a percentage of the control value.

Figure 2. Nicorandil down-regulates Ang II-induced ET-1 expression in cardiac fibroblasts. Results were shown as mean ± S.E.M. (n=6). *P<0.05 versus control; #P<0.05 versus Ang II alone; +P< 0.05 versus the nicorandil and Ang II treatment.

(A) Down-regulation of Ang II-induced ET-1 mRNA by nicorandil. Cells were preincubated with nicorandil (0.1, 0.3, 1, 3, and 10 μM) and then stimulated with Ang II (100 nM) for 30 min or not.

(B) Nicorandil inhibits Ang II-induced ET-1 secretion. Cells were preincubated with nicorandil (0.1, 0.3, 1, 3, and 10 μM) and then stimulated with Ang II (100 nM) for 24 h or not. Glibenclamide (1 μM), a K_{ATP} channel blocker, was applied in some groups.

Figure 3. Effects of nicorandil on Ang II-increased ROS formation and ERK phosphorylation. Cells were preincubated with nicorandil (3 μM) for 30 min and then stimulated with Ang II (100 nM) for 30min or not. *P<0.05 versus control; #P <0.05 versus Ang II alone.

(A) Effects of nicorandil on Ang II-induced ROS generation. Cardiac fibroblasts were treated with vehicle control (Ctrl), nicorandil (3 μM) or NAC (5 mM) for 60 min, Ang II (100 nM) for 30 min, or preincubated with nicorandil (3 μM) or NAC (5 mM) for 30 min and then stimulated with Ang II. Column bar graph of mean cell fluorescence for DCF, the fluorescence intensities in untreated control cells are expressed as 100%. Results were shown as mean ± S.E.M. (n=6).

(B) Effects of nicorandil on Ang II-increased ERK phosphorylation. Cells were preincubated with nicorandil (3 μM) or NAC (5 mM) and then stimulated with Ang II (100 nM) for 30 min. Phosphorylation of ERK was detected by Western blotting using anti-phospho-ERK antibody. Densitometric analyses were performed with a densitometer. Data was shown as fold increase relative to control groups. Results were shown as mean ± S.E.M. (n=6).

Figure 4. Effects of nicorandil on NO production, and eNOS phosphorylation. *P<0.05 versus control.

(A) Time course of nicorandil-induced NO production in cardiac fibroblasts. Cells were in control condition (Ctrl), treated with nicorandil (3 μM) for 10, 30, 60, or 120 min. Results were shown as mean ± S.E.M. (n=6).

(B) Effects of nicorandil on phosphorylation of eNOS in cardiac fibroblasts. Cells were in control condition (Ctrl), treated with nicorandil (3 μM) for 10, 30, 60, or 120 min. Western analysis performed to detect phospho-eNOS (Ser1177) and total eNOS. Results were shown as means ± S.E.M. (n=4).

Figure 5. Blockage of eNOS-NO pathway attenuated the inhibitory effect of nicorandil on Ang II–induced cell proliferation in cardiac fibroblasts. For inhibiting the activity of eNOS, the cells were pretreated with L-NAME (100 μM) for 30 min. *P<0.05 versus the mock control; #P< 0.05 versus the Ang II treatment; +P< 0.05 versus the nicorandil and Ang II treatment. Notes: Ctrl, untransfected control; M, mock control; seNOS, eNOS siRNA transfection.

(A) Effect of L-NAME, and eNOS siRNA on nicorandil-decreased Ang II-induced DNA synthesis in cardiac fibroblasts. Cells were pretreated with or without nicorandil (3 μM) for 30 min, and then treated with Ang II (100 nM) for 24 h. Results were shown as mean ± S.E.M. (n = 5).

(B) Effect of L-NAME, and eNOS siRNA on nicorandil-decreased Ang II-induced cell proliferation in cardiac fibroblasts. Cells were pretreated with or without nicorandil (3 μM) for 30 min, and then treated with Ang II (100 nM) for 24 h. Results were shown as mean ± S.E.M. (n = 6).