Mechanism of an Inhibitory Effect of Nipradilol on Rat Vascular Smooth Muscle Cell Growth

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The aim of this study was to clarify the mechanism of an inhibitory effect of nipradilol on cultured rat vascular smooth muscle cell (VSMC) growth. After being starved, cultured VSMCs were stimulated by 5% fetal bovine serum with various concentrations of nipradilol. Nipradilol dose-dependently decreased the values of [3H]-thymidine incorporation, cell numbers and total cellular protein content, and the levels of phosphorylated extracellular signal-regulated protein kinase 1/2 and p38. It also suppressed the level of proliferative cell nuclear antigen in a dose-dependent manner. In contrast, nipradilol did not change the level of the phosphorylated value of c-jun NH₂-terminal protein kinase or cytoplasmic histone-associated DNA fragments in VSMCs. These results indicate that nipradilol suppresses cell growth without apoptosis in rat VSMCs, suggesting that it could be effective for preventing the progression of restenosis after angioplasty. J Atheroscler Thromb, 2003; 10: 226–233.

Key words: Nipradilol, Vascular smooth muscle cells, MAP kinase, Proliferating cell nuclear antigen (PCNA)

Introduction

Considerable evidence supports the view that hypertension is a major risk factor for the progression of atherosclerosis and that adequate antihypertensive treatment is required to reduce the mortality and morbidity rates (1–6). The major consequence of atherosclerotic changes is the obstruction and/or occlusion of one or more arteries, leading to strokes (4) or acute coronary syndromes such as unstable angina and acute myocardial infarction (1–3, 5, 6). The mechanism(s) of arterial obstruction remains unclear, but it is generally considered that 1) increased vasoconstriction responses, 2) decreased antithrombic properties, and 3) abnormal proliferation and migration of vascular smooth muscle cells (VSMCs) are implicated in the development of atherosclerotic plaque (7). Therefore, for the prevention of atherosclerosis, it is necessary to identify the beneficial antihypertensive agents.

Nipradilol (3,4-dihydro-8-[2-hydroxy-3-isopropylamino]-propoxy-3-nitroxy-2-H-1-benzopyran), one of the most common β-adrenergic blockers, has been widely administered to hypertensive patients to reduce the prevalence of congestive heart failure (8), and myocardial ischemia (9). The agent has the structural characteristics of a benzopyran skeleton with releasing nitric oxide (NO), leading to increase guanosine 3’: 5’-cyclic monophosphate (cyclic GMP) production and subsequently relaxing tracheal smooth muscle cells (10). Interestingly, recent studies have shown that nipradilol prevents the progression of atherosclerosis through the NO-releasing action in rabbit experimental models (11, 12). These findings suggest that nipradilol treatment can restore the basal NO release by increasing the levels of cyclic GMP (11) and endothelial NO synthase mRNA (12), resulting in protecting endothelium-derived relaxation in athero-
Materials

Nipradilol was supplied by Kowa Pharmaceutical Co. (Nagoya, Japan). The following items were obtained: Dulbecco’s modified Eagle medium (DMEM) and phosphate-buffered saline (PBS) from Nissui Pharmaceutical Co. (Tokyo, Japan); fetal bovine serum (FBS) from Gibco BRL (Grand Island, NY, USA) antibodies specific or phosphospecific for extracellular-regulated protein kinase (ERK) 1/2, p38 mitogen-activated protein (MAP) kinase, c-jun NH2-terminal protein kinase (JNK) and activating transcription factor (ATF)-2, and antibodies specific for ERK 1/2, JNK, and ATF-2 from New England Biolab. (Beverly, MA, USA); antibodies specific for p38 MAP kinase, Bcl-2, and proliferative cell nuclear antigen (PCNA) from Santa Cruz Biotech. (Santa Cruz, CA, USA); [3H]-thymidine, PVDF membrane, peroxidase-conjugated immunoglobulins, and an ECL kit from Amersham (Arlington Heights, IL, USA); a cell-death detection ELISA from Roche (Manheim, Germany). All other materials were from Wako Pure Chemical (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), Bio-Rad Laboratories (Richmond, CA, USA), and Sigma Chemical (St. Louis, MO, USA).

Cell culture

VSMCs were harvested from the aortae of male Sprague-Dawley rats (150–200 g) (Funabashi Farm, Chiba, Japan) by the medial explant technique and cultured in DMEM containing 10% FBS as described previously (14). Cells within 10 passages were used for the following studies.

Measurement of DNA synthesis

The assay was performed as described previously (15). VSMCs were plated at a density of 5 × 10^4 cells per well on a 12-well culture plate and grown in DMEM containing 10% FBS for 48 h. Then, the medium was changed to DMEM containing 0.2% bovine serum albumin (BSA) for starvation. After 24 h of incubation, the media were aspirated, and the cells were cultured in DMEM containing 5% FBS with various concentrations (final concentrations: 10, 30, 50, 70, and 100 µM) of nipradilol solubilized with dimethylsulfoxide (DMSO) for 20 h. Then, one µCi of [3H]-thymidine was added to each well, and the cells were incubated for another 4 h. At the end of incubation, the cells were washed, and the labeled DNA was precipitated by 5% trichloroacetic acid at 4°C for 20 min. The precipitate was washed and solubilized in a mixture of 0.5 N NaOH and 0.1% sodium dodecyl sulfate (SDS), and the radioactivity was determined in a liquid scintillation counter. Protein concentrations were measured by the method of Lowry et al. (16).

Growth assay

1 × 10^4 of VSMCs was seeded into a 12-well culture plate and subcultured as mentioned above. After 24 h of starvation, the cells were cultured in DMEM containing 5% FBS with various concentrations (final concentrations: 30, 50, and 100 µM) of nipradilol solubilized with DMSO for the indicated number of days (0, 1, 3, or 5 days). Those media were replaced every 24 h during this experiment. Then, the media were aspirated, and the cells were washed twice with ice-cold PBS. The cells were trypsinized, and the cell numbers were counted with a Coulter counter (Coulter Electronics, Luton, UK) as described previously (17). In addition, the other cells were solubilized in 1 ml of a mixture of 0.5% SDS and 0.5 N NaOH. After the solution had been neutralized with 500 µl of 1 N HCl, the measurement of total cellular protein content was performed as described previously (15).

Immunoblot analysis of cytosolic protein

After being starved with DMEM containing 0.2% BSA for 24 h at 37°C, the cells were cultured in DMEM containing 5% FBS with various concentrations (final concentrations: 30, 50, 70, and 100 µM) of nipradilol solubilized with DMSO for another 24 hr. The following experimental procedure was carried out as described previously (18). The cells were washed and solubilized with 0.5 ml of alysis buffer consisting of 20 mM Hepes, 5 mM MgCl2, 25 mM KCl, 1 mM Na2SO4, 1 mM sodium molybdate, 10 mM β-glycerophosphate, 5 mM tetrasodium pyrophosphate, 250 mM sucrose, 1 mM adenosine 5’-triphosphate (ATP), 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mg/ml aprotinin, and 1% Triton-X. The total cellular lysate was centrifuged at × 14,000 g to remove insoluble materials, and then protein concentrations were determined by the method of Bradford (19). After being solubilized in a Laemmli buffer, the samples were sepa-
rated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes.

After being blocked overnight, the membranes were incubated with the primary anti-phosphospecific antibodies at 4°C overnight or with the antibodies at room temperature for 1 h. The blots were washed and then incubated with peroxidase-conjugated immunoglobulins at room temperature. After the blots were washed, the sites of antibody binding were visualized using the ECL Western blotting detection system and quantified with a densitometer.

**Immunoblot analysis of nuclear protein**

The cells were prepared as mentioned in the above section, and the following procedure for the extraction of nuclear protein was performed as described previously (20). After being scraped with ice-cold PBS, the cell suspension was transferred to a microfuge tube and centrifuged at ×14,000 g for 10 sec. Then, the pellets were resuspended in 400 µl of ice-cold buffer A [10 mM Hepes-KOH (pH 7.9, 4°C), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.2 mM PMSF], put on ice for 10 min, and centrifuged at 14,000 g for 10 sec. The pellets were resuspended in 50 µl of ice-cold buffer C [20 mM Hepes-KOH (pH 7.9, 4°C), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM DTT, 0.2 mM PMSF], incubated on ice for 20 min, and centrifuged at ×14,000 g for 2 min. The protein contents of these supernatants were determined by the method of Bradford (19).

The samples were solubilized in a Laemmli buffer, separated by 12% SDS-PAGE, and transferred to PVDF membranes. The blots were treated to visualize the sites of antibody binding by the same method and quantified as described in the previous section.

**Enzyme immunoassay for cytoplasmic histone-associated DNA fragments**

For the quantitative determination of apoptosis, we measured cytoplasmic histone-associated DNA fragments (mono- and oligonucleotides) using a cell-death detection ELISA as described previously (17). Briefly, the cells were plated at a density of 1×10⁴ cells per well on a 24-well culture plate and cultured in DMEM containing 10% FBS for 48 h. After being starved with DMEM containing 0.2% BSA for 24 h, the cells were cultured in DMEM containing 5% FBS with various concentrations (final concentrations: 30, 50, and 100 µM) of nipradilol solubilized with DMSO. After being incubated for 24 h, the cells were scraped and centrifuged to obtain the pellet. The cell pellet was lysed with 500 µl of the incubation buffer, and the supernatant (cytoplasmic fraction) was obtained by centrifugation at ×14,000 g for 10 min at 4°C. After 1:10 dilution, 100 µl of the samples in duplicate was added to a microtiter plate coated with anti-histone antibody and incubated for 90 min. After the samples had been washed, anti-DNA peroxidase was added to each well, and the solution was further incubated for 90 min. After being washed again, the solution containing 2,2’-azino-di-3-ethylbenzthiazoline sulfonate was added for development. The absorbance was measured at 405 nm against the substrate solution as blank as described in the above section.

**Statistical analysis**

Results are expressed as mean ± SEM. Statistical significance was estimated by the one-way analysis of variance (ANOVA) for the comparison of several groups, and the differences were considered to be significant at p < 0.05.

**Results**

**Effect of nipradilol on cell growth**

To characterize the effect of nipradilol on rat VSMC growth in vitro, we first examined the mitogenic response by a [³H]-thymidine incorporation assay. As shown in Fig. 1, the values of [³H]-thymidine incorporation were inhibited by treatment with nipradilol dose-dependently. At 50 µM, 70 µM, and 100 µM of nipradilol, the values were significantly decreased to 66%, 52%, and 32% of those of the control, respectively (p < 0.01).

To support the inhibitory effect of nipradilol on rat VSMC growth, we further examined the changes of cell number (Fig. 2). The cell numbers in the control group were gradually increased to 2.7-fold on day 1 by the stimulation of 5% FBS. Compared with those of the control, the cell numbers of the groups treated with nipradilol significantly decreased in dose-dependent manners after day 3 (p < 0.05).

![Fig. 1. Effect of nipradilol on DNA synthesis in rat VSMCs. The results were derived from four separate experiments, and each experiment was performed in triplicate. Each bar represents the mean ± SEM. *p < 0.01 vs. control.](image-url)
In addition, we also examined the changes of total cellular protein concentration (Fig. 3). The values in the control group were gradually increased to 102.7 ± 2.9 µg/ml on day 3 and 141.2 ± 2.4 µg/ml on day 5 by the stimulation of 5% FBS. Compared with the control, the values in the nipradilol-treated groups were decreased dose-dependently. The values at 30 µM of nipradilol were significantly decreased even on day 3 (p < 0.01), indicating that nipradilol itself prevented VSMC growth.

**Fig. 2.** Effect of nipradilol on the changes of growth curves in rat VSMCs. The results were derived from three separate experiments, and each experiment was performed in triplicate. Each bar represents the mean ± SEM. *p < 0.05 vs. control.

**Fig. 3.** Effect of nipradilol on the changes of total cellular protein concentrations in rat VSMCs. The results were derived from three separate experiments, and each experiment was performed in triplicate. Each bar represents the mean ± SEM. *p < 0.05, **p < 0.01 vs. nipradilol.

**Effect of nipradilol on the activation of cytosolic proteins**

Next, we used immunoblot analysis to examine whether nipradilol could affect the activation of ERK 1/2 in rat VSMCs. As shown in Fig. 4, the treatment with nipradilol decreased the phosphorylation of ERK 1/2 dose-dependently. Compared with those of the control basal band, the levels of phosphorylated ERK 1/2 were significantly suppressed to 66.7 ± 8.3% at 50 µM, to 49.5 ± 7.1% at 70 µM, and to 45.2 ± 4.5% at 100 µM (p < 0.05). The protein levels of ERK 1/2 were not changed in any of the conditions.

We examined whether nipradilol could affect the activation of stress-activated protein kinase (SAPK) 1 (also known as JNK) and SAPK 2 (also known as p38). As shown in Fig. 5 (A), the treatment with nipradilol decreased the phosphorylation of p38 dose-dependently. In comparison with the control basal band, the levels of phosphorylated p38 were significantly suppressed to 62.5 ± 8.3% at 50 µM, to 45.8 ± 5.8% at 70 µM, and to 33.3 ± 4.2% at 100 µM, which are similar results to those of ERK 1/2. The protein levels of p38 were not changed in any of the conditions. In contrast, differently from other MAP kinases such as ERK 1/2 and p38, the treatment with nipradilol did not affect the level of phosphorylation of JNK 1 [Fig. 5 (B)]. The protein levels of JNK 1 were not changed in any of the conditions.

In addition, nipradilol did not change the protein levels of Bcl-2 in rat VSMCs (data not shown).

**Fig. 4.** Effect on nipradilol on the phosphorylation and protein level of ERK 1/2 in rat VSMCs. The phosphorylation or protein level of ERK 1/2 was detected by immunoblot analysis using antibody phosphospecific or specific for ERK 1/2, as described in the Methods section. p-ERK 1/2 or ERK 1/2 indicates the phosphorylation or the protein level of ERK 1/2, respectively. The quantitation of the phosphorylated ERK 1/2 from four separate experiments is shown in the lower panel, and each bar represents the mean ± SEM. *p < 0.05 vs. control.
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Effect of nipradilol on the regulation of nuclear proteins

To estimate the inhibitory effect of nipradilol on VSMC growth in the nuclear signaling level, we investigated the expression of PCNA. Interestingly, the protein level of PCNA was dose-dependently attenuated by the treatment with nipradilol in rat VSMCs (Fig. 6). In comparison to the control band, the levels of PCNA were significantly decreased to $60.4 \pm 7.0\%$ at $50 \mu M$, to $55.8 \pm 2.4\%$ at $70 \mu M$, and to $54.8 \pm 7.1\%$ at $100 \mu M$ by the treatment with nipradilol.

Moreover, in order to confirm the identity of the effect of nipradilol on the signal transduction pathway, we further examined the phosphorylation of ATF-2 in rat VSMCs. However, the treatment with nipradilol did not affect the level of phosphorylation of ATF-2, similarly to JNK 1 (data not shown).

Effect of nipradilol on the induction of apoptosis

To evaluate an apoptotic effect of nipradilol in rat VSMCs, we examined the level of cytoplasmic histone-associated DNA fragments. As shown in Table 1, by the stimulation of 5% FBS, nipradilol could not induce cytoplasmic histone-associated DNA fragmentation.

Effect of cerivastatin on the induction of cytotoxicity

The cell viability with addition of nipradilol was more than 98% at even $100 \mu M$ after a 24-h culture, as determined by the exclusion of 0.2% Trypan blue (data not shown).

Discussion

In this study, we have clearly demonstrated that nipradilol has an inhibitory effect even on the early events of atherosclerotic formation by direct suppression of VSMC growth via an ERK pathway and even regulation of a cell cycle in a strict dose-dependent manner. These favorable results suggest that nipradilol could be one of the most promising agents for preventing the progression of vascular remodeling such as restenosis after percutaneous transluminal coronary angioplasty (PTCA).

We first examined the effect of nipradilol on the proliferation of rat VSMCs. The values of $[\text{H}]$-thymidine incorporation and the changes of total protein content and cell numbers were specifically reduced by the addition of nipradilol in dose-dependent fashions (Figs. 1, 2, and 3). In addition, nipradilol did not affect the cell viability, indicating that niprodilol itself can inhibit VSMC proliferation without causing cytotoxicity. In agreement with our findings, Kitaoka et al. have reported that niprodilol dose-dependently alter the relaxant effect of the canine isolated posterior ciliary artery at more than $100 \mu M$ without any significant cytotoxicity (21).

Recently, it was revealed that the MAP kinase superfamily plays a crucial role in cell growth, differentiation, or even programmed cell death in response to diverse extracellular stimuli in eukaryotic cells (22). Extensive studies have clarified that a variety of growth factors and hormones can activate the ERK 1/2 (p44/42) signal transduction pathways through the GTPase-activating protein

![Fig. 5. Effect of nipradilol on the phosphorylation and protein level of p38 (A) and JNK 1 (B) in rat VSMCs. The phosphorylations or protein levels of p38 and JNK 1 were detected by immunoblot analysis using antibodies phosphospecific or specific for p38 and JNK 1, as described in the Methods section. P-p38 and p-JNK 1 or p38 and JNK 1 indicate the phosphorylations or the protein levels of p38 and JNK 1, respectively. The quantitation of the phosphorylated p38 or JNK 1 from four separate experiments is shown in the lower panel, and each bar represents the mean ± SEM. *p < 0.05, **p < 0.01 vs. control.](image)

![Fig. 6. Effect of nipradilol on the protein level of PCNA in rat VSMCs. The level of PCNA was detected by immunoblot analysis using antibody specific for PCNA, as described in the Methods section. The quantitation of the level of PCNA from four separate experiments is shown in the lower panel, and each bar represents the mean ± SEM. *p < 0.05 vs. control.](image)
of Ras (Ras-GTP), leading to cellular proliferation and differentiation by stimulating transcription factors that induce the expression of c-fos and other growth-responsive genes (23, 24). Therefore, we next examined whether nipradilol could affect the activation of various MAP kinases in rat VSMCs by immunoblot analysis. As shown in Figure 4, nipradilol specifically inhibited the levels of phosphorylated ERK 1/2 in dose-dependent fashions and significantly reduced the levels at more than 50 µM, suggesting that nipradilol may have an effect on the change(s) of signal transduction through Ras-GTP to an ERK pathway. Moreover, we examined whether nipradilol could affect the activation of p38 and JNK. As shown in Figure 5 (A), the level of phosphorylated p38, as well as those of ERK 1/2, was also inhibited dose-dependently by the agent in the same fashion. Although we did not examine the biochemical consequence of phosphorylated p38 in detail in this study, we and other investigators have recently reported that the activated p38 plays a crucial role in the regulation of VSMCs. Matsumoto et al. have shown that platelet-derived growth factor-BB activates p38 through a Ras-dependent pathway that plays an important role in actin reorganization and cell migration (25). In addition, we have shown that insulin-induced p38 activation partly regulates VSMC growth, independently of an ERK pathway (26). Therefore, it is reasonable to assume that the inhibition of phosphorylated p38 by nipradilol might result in the suppression of VSMC growth and/or migration. On the other hand, differently from ERK 1/2, the activation of JNK led to apoptosis in response to extracellular stimuli in various types of cells (27, 28), and some anti-atherosclerotic agents such as statins can simultaneously inhibit cell growth and induce apoptosis in VSMCs from various species (17, 29). As shown in Figure 6 (B), nipradilol could not induce the phosphorylation of JNK 1 in rat VSMCs. We further examined the changes of the protein level of Bcl-2 and cytoplasmic histone-associated DNA fragmentation, which are more reliable apoptosis markers (17, 30), by the treatment of nipradilol in rat VSMCs. However, nipradilol did not affect the protein levels of Bcl-2 (data not shown) or the values of cytoplasmic histone-associated DNA fragmentation (Table 1), suggesting that the treatment with nipradilol may not result in apoptosis in rat VSMCs.

Although the precise molecular mechanism(s) by which nipradilol can affect the ERK pathway remains unclear, one likely contributor is the changes in intracellular calcium concentration in rat VSMCs, since Abe et al. have shown that the agent, at high dosage, can reduce the intracellular calcium concentration in pig coronary artery (13). In addition, we and other investigators have revealed that the changes in intracellular calcium concentration have an important role in regulating ERK activation in the various types of cells (15, 31, 32). Lucchesi et al. have reported that angiotensin II stimulates ERK 1/2 phosphorylation in a calcium-dependent manner in rat VSMCs and that the phosphorylation is stronger in spontaneously hypertensive rats that in Wistar-Kyoto rats (31). We have also recently shown that nifedipine, one of the most common calcium antagonists, can inhibit rat VSMC proliferation via the ERK pathway coupling with Pyk2 (15), which activates the ERK pathway in response to the elevation of intracellular calcium concentrations (33). These findings may support the possibility that nipradilol directly prevents cell proliferation by inhibiting calcium accumulation in rat VSMCs.

To evaluate the effects of nipradilol on the regulation of the nuclear level of VSMCs, we examined the levels of PCNA, which were synthesized in the early G1 and S phases of the cell cycle and behave as a marker for proliferating cells (34, 35). In this study, we could show that nipradilol can dose-dependently suppress the level of PCNA (Fig. 6), suggesting that nipradilol can inhibit VSMC growth by arresting in the G1 phase of the cell cycle. In contrast, we could not observe any increase of phosphorylated ATF-2, a transcription factor found in the downstream of JNK (22, 27) (data not shown), which demonstrates that nipradilol has the possibility to prevent VSMC growth by the reduction of the ERK cascade but not by the induction of apoptosis.

In conclusion, in this study, we have clearly characterized the cellular mechanism of the inhibitory effects of nipradilol on rat VSMC growth. Nipradilol suppressed the proliferative cellular signaling in VSMCs, suggesting that it could be effective for preventing the progression of atherosclerotic plaque, such as restenosis after PTCA. These results provide a new insight into the potential cellular and molecular mechanisms whereby nipradilol treatment may have clinical benefits and contribute to the prevention of atherosclerosis.

**References**

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| Relative fold of control DNA fragmentation | 5% FBS |
|--------------------------------------------|--------|
| Control                                    | 1      |
| Nipradilol       |        |
| 30 µM          | 1.02 ± 0.08 |
| 50 µM          | 1.08 ± 0.09 |
| 100 µM         | 1.16 ± 0.13 |

The results were derived from three separate experiments, and each experiment was performed in triplicate. Values are the mean ± SEM.
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