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ORIGINAL

Fasudil hydrochloride induces osteoblastic differentiation of stromal cell lines, C3H10T1/2 and ST2, via bone morphogenetic protein-2 expression

Ippei Kanazawa, Toru Yamaguchi, Shozo Yano, Mika Yamauchi and Toshitsugu Sugimoto

Department of Internal Medicine 1, Shimane University Faculty of Medicine, Izumo 693-8501, Japan

Abstract. Rho-kinase (ROK), downstream of the mevalonate pathway, is detrimental to vessels, and suppressing its activity is a target for the treatment of human disease such as coronary artery disease and pulmonary hypertension. Recent studies have shown that ROK has a crucial role in bone metabolism. However, the role of ROK in stromal cells is still unclear. The present study was undertaken to investigate the effect of a ROK inhibitor, fasudil hydrochloride, on stromal cell lines, C3H10T1/2 and ST2. In both cells, fasudil significantly stimulated alkaline phosphatase activity and enhanced cell mineralization. Moreover, fasudil significantly increased the mRNA expression of collagen-I, osteocalcin, and bone morphogenetic protein-2 (BMP-2). Supplementation of noggin, a BMP-2 antagonist, significantly reversed the fasudil-induced collagen-I and osteocalcin mRNA expression in both cells. These findings suggest that fasudil induces the osteoblastic differentiation of stromal cells via enhancing BMP-2 expression, and that this drug might be beneficial for not only atherosclerosis but also osteoporosis by promoting bone formation.

Key words: Fasudil, Rho kinase, BMP-2, Stromal cell, Bone formation

HMG-CoA reductase inhibitors (statins) are widely used as cholesterol-lowering medicines for the prevention of coronary heart disease. Moreover, statins have recently been shown to exert pleiotropic effects on various cells, which may not be directly related to cholesterol synthesis. The small GTPase Rho and one of its downstream effectors, the Rho-kinase (ROK), which are located in the downstream of HMG-CoA reductase, have attracted widespread attention as the most important factors of pleiotropic effects by statins. ROK has intensely been investigated about its detrimental effects on the cardiovascular disease [1], and has been implicated in many of the pathologic processes that underlie atherosclerosis including endothelial dysfunction, vasoconstriction, inflammation, cellular migration and proliferation, and a procoagulant state [2, 3]. Indeed, fasudil hydrochloride, a specific inhibitor of ROK, effectively treats a wide range of the cardiovascular disease in several clinical studies [4]. On the other hand, we and other investigators have recently shown that fasudil could also affect bone by inducing bone morphogenetic protein-2 (BMP-2) in osteoblasts and stimulate their differentiation and mineralization [5, 6]. Thus, agents inhibiting ROK are expected to be candidate drugs not only curing the cardiovascular disease but also promoting bone formation for the treatment of osteoporosis.

Osteoblasts and adipocytes are known to originate from a common progenitor, which arises from bone marrow stromal cells. The status of stem cells changes with respect to both their intrinsic differentiation potential and production of signaling molecules, which contributes to the formation of a specific marrow microenvironment necessary for maintenance of bone homeostasis. However, to our knowledge, there are no reports to investigate whether or not ROK inhibition would affect osteoblast lineage commitment on stromal cells. Therefore, to investigate this issue, we specifically examined the effects of fasudil, a specific ROK inhibitor, on the differentiation of stromal cells by using its cell lines, C3H10T1/2 and ST2.

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Correspondence to: Ippei Kanazawa, Department of Internal Medicine 1, Shimane University Faculty of Medicine, 89-1 Enya-cho, Izumo 693-8501, Japan. E-mail: ippei.k@med.shimane-u.ac.jp

Abbreviations: ROK, Rho-kinase; BMP-2, bone morphogenetic protein-2; ALP, Alkaline phosphatase

Materials and Methods

Materials

Cell culture medium and supplements were purchased from Gibco-BRL (Rockville, MD). Fasudil was a kind gift from Asahi-kasei Corporation (Tokyo, Japan). Noggin, a BMP antagonist, was purchased from PeproTech Inc. (Rocky Hill, NJ). All other chemicals were of the highest grade available commercially.

Cell cultures

C3H10T1/2 and ST2 cells, murine stromal cell lines, were kindly provided by Dr. H. Kaji, Kobe University. C3H10T1/2 cells were grown in Eagle's basal medium, and ST2 cells were cultured in α-minimum essential medium (α-MEM). These medium were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (GIBCO-BRL) in 5% CO_2 at 37°C and changed twice a week. For mineralization assay, these cells were cultured in each medium containing 10% FBS, 1% penicillin-streptomycin, 5mM β-glycerophosphate, 100 μg/mL ascorbic acid, and 50 ng/mL BMP-2 after reaching confluence.

Real-time PCR quantification of gene expression

SYBR green chemistry was used to perform quantitative determinations of the mRNAs for BMP-2, collagen-I, osteocalcin, and a house keeping gene, 36B4, according to an optimized protocol [6-9]. Total RNA was taken from cultured C3H10T1/2 and ST2 cells using Trizol reagent (Invitrogen, San Diego, CA) according to the manufacturer's recommended protocol. Two μg total RNA was employed for the synthesis of single-stranded cDNA (cDNA synthesis kit; Invitrogen). The sense and antisense primers were designed using the Primer Express Version 2.0.0 (Applied Biosystems Inc.) based on published cDNA sequences. The double-stranded DNA-specific dye SYBR Green I was incorporated into the PCR buffer provided in the QuantiTech SYBR PCR kit (QIAGEN, Valencia, CA) to allow for quantitative detection of the PCR product. The temperature profile of the reaction was 95°C for 15 min, 40 cycles of denaturation at 94°C for 15 sec, and annealing and extension at 60°C for 1 min. 36B4 was used to normalize differences in RNA isolation, RNA degradation, and the efficiencies of the reverse transcription.

Assay of alkaline phosphatase activity

After reaching confluence, cells in 24-well plates were rinsed three times with PBS, and 600 μL of distilled water was added to each well and sonicated. The protein assay was performed with the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL). Alkaline phosphatase (ALP) activity was assayed by a method modified from that of Lowry *et al*. [10]. In brief, the assay mixtures contained 0.1 M 2-amino-2-methyl-1-propanol, 1 mM $MgCl_2$, 8 mM p-nitrophenyl phosphate disodium, and cell homogenates. After a 4 min of incubation at 37°C, the reaction was stopped with 0.1N NaOH, and the absorbance was read at 405 nm. A standard curve was prepared with p-nitrophenol. Each value was normalized to the protein concentration.

Assay of mineralization

Mineralization of ST2 cells was determined in 6-well or 12-well plates using von Kossa staining or Alizarin red staining. The cells were stained with 2% $AgNO_3$ and fixed with 2.5% NaS_2O_3 by von Kossa method to detect phosphate deposits in bone nodules [6-8]. At the same time, the order plates were fixed with ice-cold 70% ethanol and stained with Alizarin red to detect calcification. For quantification, cells stained with Alizarin red were destained with ethylpyridium chloride, then the extracted stain was transferred to a 96-well plate, and the absorbance at 550 nm was measured using a microplate reader, as previously described [6-8].

Statistics

Results are expressed as a mean ± SEM. Statistical evaluations for differences between groups were carried out using ANOVA followed by Fisher's protected least significant difference (PLSD). For all statistical tests, a value of $P < 0.05$ was considered to be a statistically significant difference.

Results

The effect of fasudil on the differentiation and mineralization of stromal cells

We first investigated whether or not fasudil could augment the osteoblastic differentiation of C3H10T1/2 and ST2 cells. Fasudil (10^{-6} and 10^{-5} M) significantly augmented ALP activity on 10 days in C3H10T1/2 cells (Fig. 1A) and ST2 cells (Fig. 1B) as well as min-

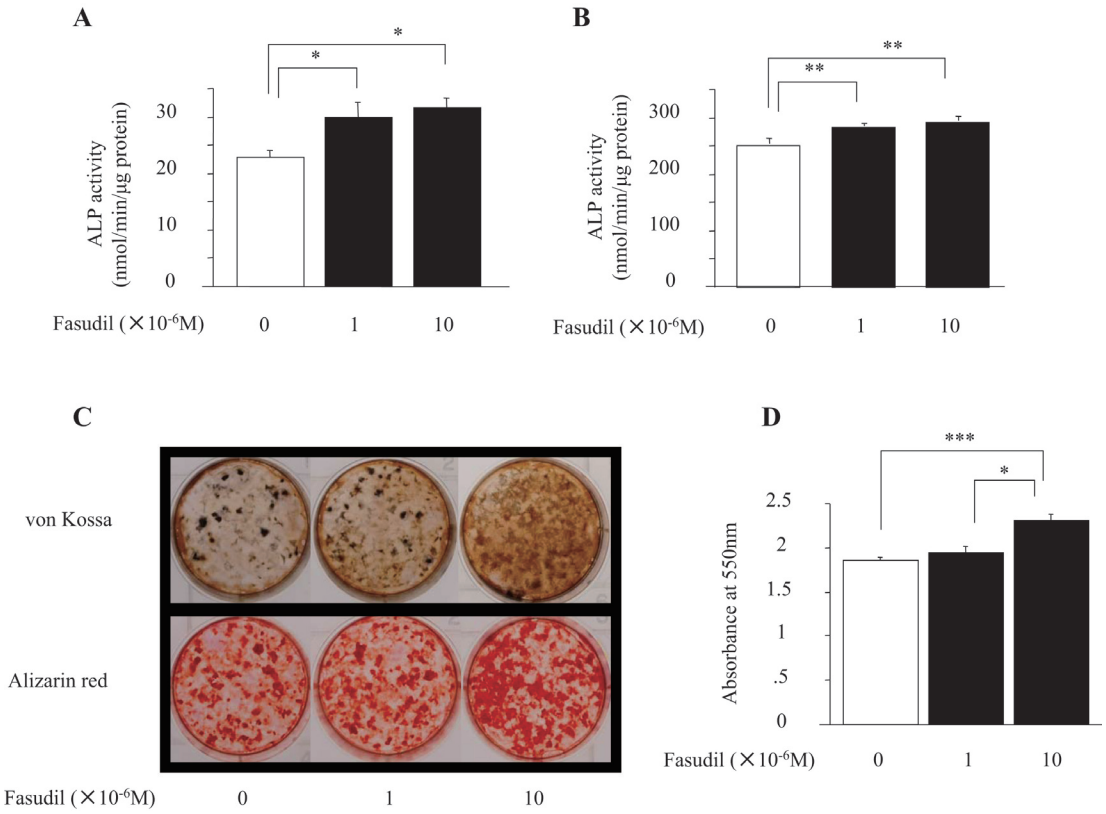

Fig. 1. The effect of fasudil on the osteogenic differentiation of stromal cells.
The ALP activities were augmented by fasudil (10^{-6} and 10^{-5} M) in C3H10T1/2 cells (A) and ST2 cells (B) on 10 days. A plate view of von Kossa and Alizarin red stainings in cultured ST2 cells (C). Quantification of Alizarin red staining via extraction with etylpyridinium chloride (D). Mineralization was augmented by fasudil by both von Kossa and Alizarin red stainings. Quantification of Alizarin red staining showed that the effects of 10^{-6} M and 10^{-5} M fasudil on the mineralization were significant. The result was the representative of three different experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

eralization of ST2 cells on 21 days by both von Kossa and Alizarin red stainings (Fig. 1C) and by Alizarin red quantification (Fig. 1D) in dose-dependent manners (at least $p<0.05$).

The effect of fasudil on BMP-2 mRNA expression in stromal cells

Next, we examined whether or not fasudil could stimulate BMP-2 mRNA expression in C3H10T1/2 and ST2 cells. Fasudil was added after the cells reached confluency on 7 days, and total RNA was collected after 12 and 24 hours. Fasudil (10^{-5} M) significantly increased BMP-2 mRNA expression at 24 hours by real-time PCR in C3H10T1/2 cells (Fig. 2A) and ST2 cells (Fig. 2C) ($p<0.05$). Fasudil (10^{-6} and 10^{-5} M) also significantly increased BMP-2 mRNA expression at 24 hours in dose-dependent manners in C3H10T1/2 cells (Fig. 2B) and ST2 cells (Fig. 2D) (at least $p<0.05$).

The effects of a BMP antagonist on the fasudil-induced differentiation in stromal cells

We investigated whether or not BMP-2 was actually involved in the fasudil-induced differentiation in C3H10T1/2 and ST2 cells by inhibiting its activity. Fasudil (10^{-5} M) significantly increased osteocalcin and collagen-I α1 mRNA expression at 24 hours in C3H10T1/2 cells (Figs. 3A and 3B, respectively) and ST2 cells (Figs. 3C and 3D, respectively) ($p<0.05$). Co-incubation with 100 ng/mL noggin, a BMP antagonist, significantly reversed the fasudil-induced enhancement of osteocalcin mRNA expression in C3H10T1/2 cells (Fig. 3A) and ST2 cells (Fig. 3C) ($p<0.05$) as well as that of collagen-I mRNA expression in C3H10T1/2 cells (Fig. 3B) and ST2 cells (Fig. 3D) (at least $p<0.01$).

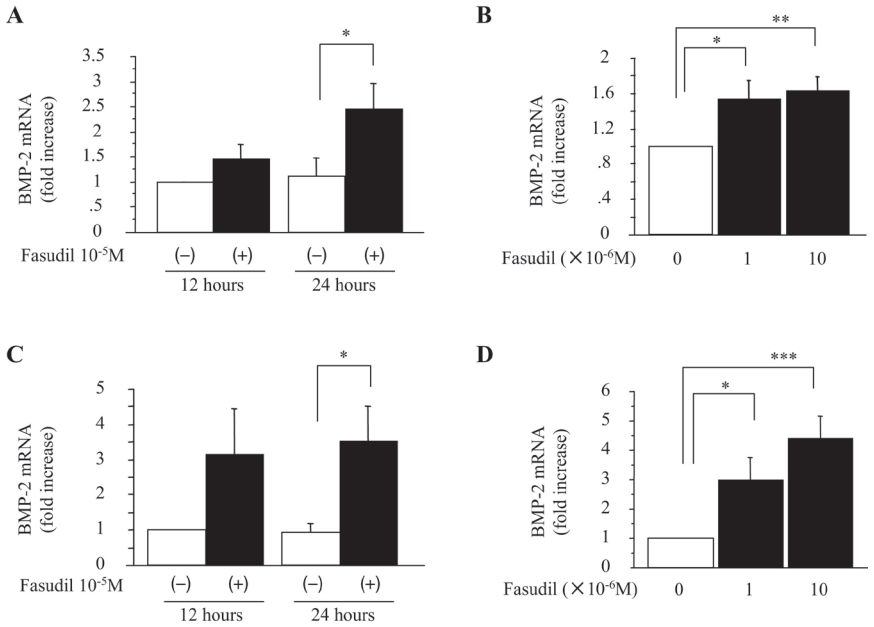

Fig. 2. The effect of fasudil on BMP-2 mRNA expression in stromal cells.
BMP-2 mRNA expression was significantly increased by fasudil (10^{-5} M) at 24 hours in C3H10T1/2 cells (A) and ST2 cells (C). Fasudil (10^{-6} and 10^{-5} M) stimulated BMP-2 mRNA expression at 24 hours in dose-dependent manners in C3H10T1/2 cells (B) and ST2 cells (D). Results are expressed as the mean ± SEM fold increase over control values (n = 9). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

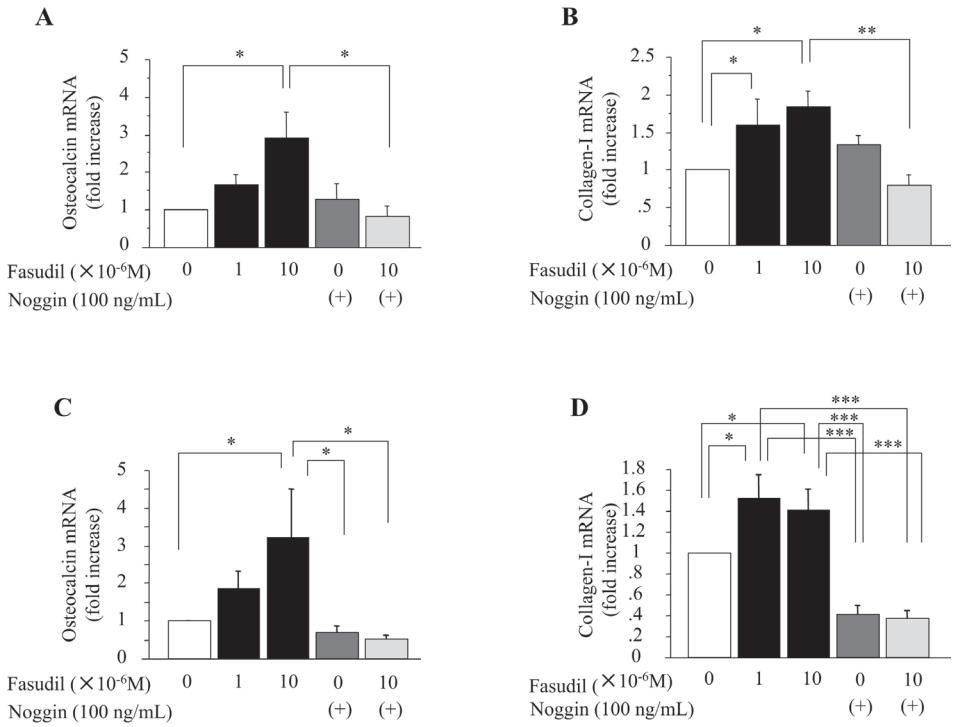

Fig. 3. The effects of a BMP antagonist on the fasudil-induced differentiation in stromal cells.
Fasudil (10^{-5} M) stimulated osteocalcin and collagen-I mRNA expression at 24 hours in C3H10T1/2 cells (A and B) and ST2 cells (C and D). Fasudil-augmented osteocalcin and collagen-I mRNA expression was reversed by the addition of 100 ng/mL noggin, a BMP-2 antagonist, in both cells (A-D). Results are expressed as the mean ± SEM fold increase over control values (n = 9). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Discussion

In this study, we demonstrated for the first time that fasudil, a specific ROK inhibitor, augmented osteoblastic differentiation as well as mRNA expression of BMP-2, osteocalcin, and collagen-I in stromal cell lines. Co-incubation with noggin, a BMP-2 antagonist, significantly reversed the fasudil-induced increase in osteocalcin and collagen-I mRNA expression. Thus, fasudil seems to promote osteoblastic differentiation of stromal cells via an increased production of BMP-2 in autocrine or paracrine fashions.

ROK has recently attracted widespread attention due to their beneficial anti-atherosclerotic effects. Accumulating evidence has demonstrated that the Rho/ROK pathway plays an important role in various cellular functions [4]. At a molecular level, ROK upregulates various molecules that accelerate inflammation/oxidative stress and fibrosis, whereas it downregulates NOS. ROK activity is increased in animals with experimental atherosclerosis [11, 12], hypertension [13], and diabetes [14]. Furthermore, risk factors for atherosclerosis including oxidized LDL [15], hyperglycemia [16], and nicotine [17] increase Rho/ROK activity. On the other hand, there are only a few reports to investigate the role of ROK in osteoblasts. Harmey *et al.* [18] showed that activation of Rho decreased differentiation and mineralization of primary mouse calvarial osteoblasts and reduced gene expression of osteoblastic markers, including BMP-2, while chemical inhibition of ROK enhanced osteoblastic markers as well as bone nodule formation. We have recently shown that inhibition of ROK by fasudil induced BMP-2 expression and enhanced differentiation and mineralization of osteoblastic MC3T3-E1 cells [6]. In this study, we also found that fasudil stimulated the differentiation of stromal cell lines via BMP-2 expression, although we have not performed time-dependent experiments or examined its protein expression. Thus, the Rho/ROK pathway seems to be an important negative regulator of bone formation, which inhibits osteoblast lineage commitment on stromal cells. However, Meyers *et al.* [19] showed that overexpression of constitutively active RhoA enhanced the osteoblastic phenotype such as increased gene expression of type I collagen, ALP, and runt-related transcription factor 2, and suppressed the adipocytic phenotype of human stromal cells cultured in modeled microgravity. In this study, we found that fasudil stimulated osteoblastogenesis of both cell lines only at higher concentrations (10^{-6} and 10^{-5} M). Therefore, further studies are needed to clarify whether the Rho/ROK pathway would play stimulatory or inhibitory roles in the differentiation of stromal cells. In addition, Liu *et al.* reported that activation of Smad 1/5/8, which are located in the downstream of BMP-2 signaling, was mediated by Rho/ROK in pulmonary artery smooth muscle cells [20]. Therefore, further studies are also needed to examine the interaction of ROK with Smads in stromal cells.

BMPs, a group of polypeptides within the transforming growth factor (TGF)-β superfamily, were originally identified by their ability to induce endochondral bone formation in ectopic extraskeletal sites *in vivo* [21]. BMPs stimulate differentiation of pluripotent stem cells into the osteogenic lineage and enhance the differentiated function of osteoblasts. Among many other BMPs, BMP-2 induces differentiation of preosteoblasts into mature osteoblasts by regulating signals that stimulate a specific transcriptional program required for bone formation [22, 23]. BMP-2 stimulates expression of Runx2, the only known osteoblast-specific transcription factor to induce genes required for mature osteoblast differentiation and maintenance [24, 25]. Therefore, BMP-2 and agents stimulating its expression are expected for treatments of osteoporosis and bone fractures. The present study shows that fasudil could also stimulate bone formation through BMP-2 production in autocrine or paracrine fashions.

Many clinical studies have shown that osteoporosis is associated with the cardiovascular disease and influences mortality [26-30]. Although both diseases are traditionally viewed as separate disease entities that increase in prevalence with aging, accumulating evidence indicates that there are similar pathophysiological mechanisms underlying them. In human endothelial cells *in vitro*, activation of ROK results in reduced eNOS expression and activity, which are reversed by the addition of fasudil [31]. Rho/ROK is also known to be a target of statins [32]. Inhibition of Rho/ROK by statins augments eNOS expression via stabilization of eNOS mRNA [33]. Our findings of the anabolic effect of the ROK inhibitor on bone suggest that ROK is not only a powerful mediator of endothelial dysfunction but also might be a key molecule connecting with osteoporosis and the cardiovascular disease. Thus, agents inhibiting ROK activity might be candidate drugs not only curing the cardiovascular disease but also promoting bone formation for the treatment of osteoporosis.

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