



Evidence of a direct cellular protective effect of Rho-kinase inhibitors on endothelin-induced cardiac myocyte hypertrophy

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ABSTRACT

Using a cellular approach, the present study examined whether fasudil and active metabolite hydroxyfasudil, Rho-kinase inhibitors, exert a direct protective effect on endothelin-induced cardiac myocyte hypertrophy in vitro. Treatment with endothelin (10 nM) caused significant hypertrophy of cultured neonatal rat cardiomyocytes by a 21.2% increase in cell surface area. Fasudil (1–10 μM) and hydroxyfasudil (0.3–10 μM) significantly prevented endothelin-induced cardiomyocyte hypertrophy. The present results suggest that inhibition of cardiac hypertrophy by fasudil is, at least in part, due to direct protection of cardiomyocytes from hypertrophy.

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1. Introduction

Cardiac hypertrophy is recognized as an adaptive increase in heart size characterized by the growth of individual cardiomyocytes rather than an increase in cell number. Cardiac hypertrophy is induced by a variety of diseases, including hypertension, pulmonary arterial hypertension, valvular diseases, myocardial infarction, and endocrine disorders [1,2]. Although cardiac hypertrophy has been considered a compensatory mechanism required to normalize wall tension and to maintain cardiac output, prolonged hypertrophy is a leading cause of heart failure and sudden death.

Growth-promoting factors such as angiotensin II and endothelin have been identified as triggers of a hypertrophic response at the cardiomyocyte level. It has been reported that Rho/Rho-kinase is involved in the signal transduction pathway in angiotensin II-induced and endothelin-induced cardiac hypertrophy [3,4]. To date, the only Rho-kinase inhibitor employed clinically in humans is fasudil, which has been used for the prevention of cerebral vasospasm and subsequent ischemic injury after surgery for subarachnoid hemorrhage [5]. In a previous study of rat in vivo, fasudil suppressed the development of angiotensin II-induced cardiac hypertrophy [6]. Because Rho-kinase is involved in various cellular functions, including vascular smooth muscle contraction, down-regulation of endothelial nitric oxide synthase, and cell migration

[7], the inhibitory effect of Rho-kinase inhibitors on cardiac hypertrophy in vivo seems to be mediated by multiple mechanisms.

Using a cellular approach, the present study examined whether fasudil and active metabolite hydroxyfasudil, another Rho-kinase inhibitor, exert a direct protective effect on endothelin-induced cardiac myocyte hypertrophy in vitro.

2. Materials and methods

All animals were used in accordance with ethical procedures approved by the Japanese Pharmacological Society for the care and use of laboratory animals.

2.1. Cardiomyocyte culture

Neonatal rat ventricular myocytes were prepared from the hearts of 1- to 2-day-old Sprague–Dawley rats. Myocardial cells, which were seeded into dishes at a density of 3×10^4 cells/cm², were incubated in 10% fetal calf serum containing DMEM/F12 at 37 °C for 24 h, and then further incubated with DMEM/F12 without serum for 48 h.

2.2. Cell treatment

Cells were treated with or without fasudil (0.3–10 μM) or hydroxyfasudil (0.3–10 μM). After 1 h of treatment with fasudil or hydroxyfasudil, cells were treated with or without endothelin (10 nM). After incubation with or without fasudil, hydroxyfasudil, and/or endothelin for 48 h, photomicrographs were obtained.

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2.3. Measurement of cell surface area

The surface area of the cardiomyocytes was determined in cells before and after intervention with drugs. The cell surface area was measured using a computerized image analysis system (NIH Image 1.61J) from 60 randomly selected cells per experiment and averaged to give an *N* value of 1.

2.4. Statistics

Values are expressed as means \pm SEM. A statistical analysis of the data was done with Student's *t* test or Dunnett's test. *P* values of 0.05 or less were considered to indicate a significant difference.

3. Results

Representative photomicrographs of neonatal rat cardiomyocytes cultured for 48 h with or without fasudil and/or endothelin are provided in Fig. 1. As shown in Fig. 1D, endothelin caused cardiomyocyte hypertrophy. Fasudil prevented the endothelin-induced cardiomyocyte hypertrophy (Fig. 1F).

The surface area of the cardiomyocytes before intervention with drugs was defined as 100%. A quantitative analysis showed that treatment with endothelin (10 nM) caused significant hypertrophy of the cardiomyocytes by a 21.2% increase in cell surface area

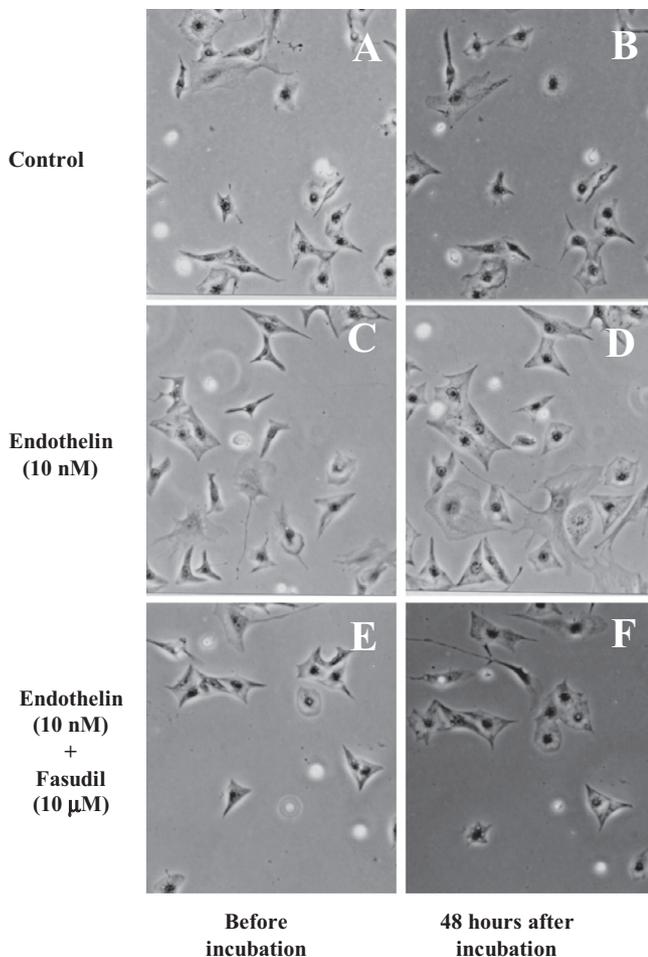


Fig. 1. Representative photomicrographs of cardiomyocytes cultured for 48 h with or without fasudil (10 μ M) and/or endothelin (10 nM). Before intervention with drugs (A, C, E). After incubation with or without fasudil and/or endothelin for 48 h (B, D, F).

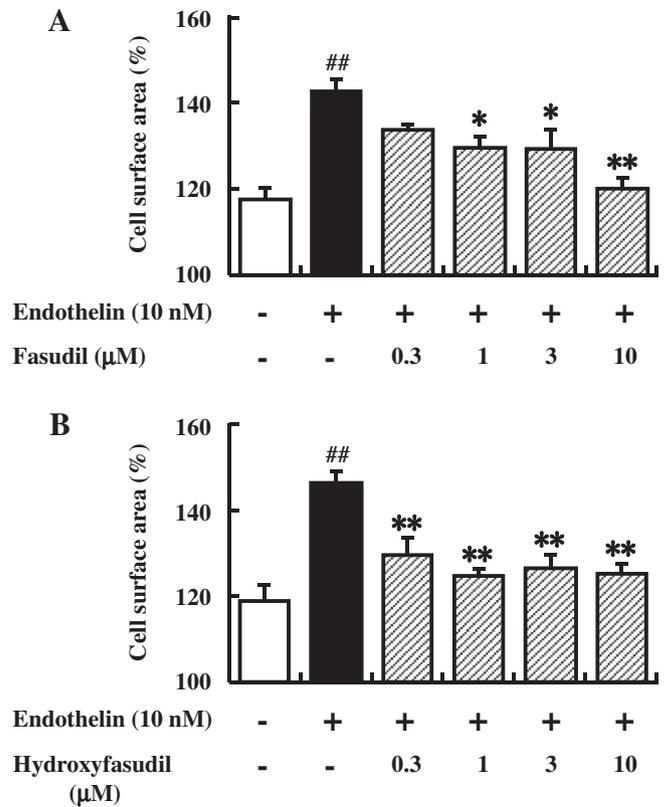


Fig. 2. Inhibition of endothelin-induced cardiomyocyte hypertrophy by fasudil (A) or hydroxyfasudil (B). The surface area of cardiomyocytes before intervention with drugs was defined as 100%. Fasudil (1–10 μ M) and hydroxyfasudil (0.3–10 μ M) significantly prevented the endothelin-induced cardiomyocyte hypertrophy. Each column represents the mean \pm SEM of 3 experiments. ##*p* < 0.01 vs. control (non-treated) group and **p* < 0.05, ***p* < 0.01 vs. endothelin-treated group, respectively.

(control (non-treated) group, 117.5 \pm 2.9%; endothelin-treated group, 142.4 \pm 3.0%) (Fig. 2A). Fasudil (1–10 μ M) significantly prevented the endothelin-induced cardiomyocyte hypertrophy. For example, fasudil (10 μ M) held the cell surface area to 120.1 \pm 2.5% in contrast to endothelin-treated cell surface area of 142.4 \pm 3.0% (*P* < 0.01) (Fig. 2A). Hydroxyfasudil (0.3–10 μ M) also significantly prevented endothelin-induced cardiomyocyte hypertrophy (Fig. 2B).

4. Discussion

Although fasudil has shown effectiveness on cardiac hypertrophy in a series of in vivo experimental studies [6,8], its cellular mechanism of action has not been fully elucidated. Accumulating evidence indicates that fasudil and hydroxyfasudil have a broad spectrum of pharmacological properties including inhibition of vascular constriction [9] and endothelial damage due to inhibition of tissue factor expression, endothelial hyperpermeability [10] and superoxide production [6], and due to upregulation of endothelial nitric oxide synthase activity [11], as well as inhibition of hyperviscosity [12] and inflammatory responses via inhibition of neutrophil and monocyte infiltration [13,14]. In the present study, to exclude the possibility of indirect modulation via systemic effects on cardiac hypertrophy, cultured cardiomyocytes were used to examine whether fasudil and hydroxyfasudil exert a direct protective effect on cardiac myocyte hypertrophy. Fasudil and hydroxyfasudil show a preventive effect on the development of cardiomyocyte hypertrophy in vitro, and this finding indicates that treatment with fasudil has therapeutic benefits against cardiac hypertrophy through not

only indirect systemic effects but also a direct effect on cardiomyocytes.

Previous studies have shown the possibility that agents with a negative inotropic effect on the heart are useful for the treatment of cardiac hypertrophy [15]. Because fasudil and hydroxyfasudil have shown no inotropic effect on isolated hearts of guinea pigs at concentrations up to 10^{-4} M [16,17], a protective effect on cardiac myocyte hypertrophy by fasudil and hydroxyfasudil is not attributable to negative inotropism.

Fasudil is metabolized to the active metabolite hydroxyfasudil in humans. In patients with subarachnoid hemorrhage, hydroxyfasudil was found in patients following intravenous infusion of fasudil (30 mg/30 min), and the area under the plasma concentration–time curve (AUC) value of hydroxyfasudil was 4.5 times higher than the value of fasudil [18]. In rats, hydroxyfasudil was also found following an oral administration of fasudil at 10 mg/kg, and the AUC value of hydroxyfasudil was approximately 20 times higher than that of fasudil [19]. Present findings showed fasudil and hydroxyfasudil are equipotent in the inhibition of cardiomyocyte hypertrophy, and indicate that hydroxyfasudil contributes to the potency of fasudil in inhibiting cardiac hypertrophy in vivo.

The present findings show that fasudil and hydroxyfasudil prevent the hypertrophy of cultured neonatal rat cardiomyocytes in vitro. These results suggest that in vivo inhibition of cardiac hypertrophy by fasudil is, at least in part, due to direct protection of cardiomyocytes from hypertrophy.

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