FULL PAPER *Pharmacology*

Endostatin inhibits bradykinin-induced cardiac contraction

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ABSTRACT. Endogenous fragments of extracellular matrix are known to possess various biological effects. Levels of endostatin, a fragment of collagen type XVIII, increase in certain cardiac diseases, such as cardiac hypertrophy and myocardial infarction. However, the influence of endostatin on cardiac contraction has not been clarified. In the present study, we investigated the effects of endostatin on bradykinin-induced atrial contraction. Isometric contractile force of mouse isolated left atria induced by electrical current pulse was measured. Voltage-dependent calcium current of guinea pig ventricular myocytes was measured by a whole-cell patch-clamp technique. Endostatin (100–1,000 *n*g/m*l*) alone treatment had no influence on left atrial contraction. On the other hand, pretreatment with endostatin (300 *n*g/m*l*) significantly inhibited bradykinin (1 μM)-induced contraction and voltage-dependent calcium current. These data suggest that endostatin may decrease bradykinin-induced cardiac contraction perhaps through the inhibition of voltage-dependent calcium channel.

KEY WORDS: bradykinin, bradykinin type 2 receptor, endostatin, voltage-dependent calcium channel

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Angiotensin converting enzyme (ACE) inhibitors are widely used for the treatment of hypertension and heart failure. Because ACE catalyzes the degradation of bradykinin, prolonged ACE inhibitor treatment increases bradykinin level [17]. Bradykinin binds to two kinds of receptors, B_1 receptor (R) and B_2R. In pathological conditions, B_1R expression is up-regulated, that is related to the inflammatory responses or pain-producing effects [1, 8, 9]. On the other hand, B_2R is widely distributed in the systemic organs, one of the main functions of which is to mediate vasodilation [9]. Because a B_2R inhibitor exacerbated left ventricular hypertrophy in rats with aortic banding [11] and in dogs with transmyocardial direct current shock [12], it is believed that bradykinin has cardioprotective action through the B_2R [1].

Endostatin, a non-collagenous fragment of collagen type XVIII [5, 13, 23], has various biological effects, such as anti-angiogenic and anti-carcinogenic effects [2–4, 10, 20, 24]. It has been reported that levels of endostatin increased in animal models of cardiac hypertrophy and post-myocardial infarction [6, 7]. While the roles of endostatin on cardiac diseases still remain unclear, we have recently reported that endostatin stimulated proliferation and migration of cardiac fibroblasts [15]. Therefore, it is suggested that endostatin might have various biological functions in heart tissues. However, the influence of endostatin on cardiac contraction has not been clarified.

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It was recently reported that regulation of Ca^{2+} homeostasis through B_2R plays an important role in the cardiac contraction [18]. Specifically, bradykinin was shown to mediate the opening of Ca^{2+} channels through B_2R in guinea pig cardiomyocytes [19]. Therefore, it is also suggested that bradykinin plays a role in physiological myocardial contraction. While endostatin was shown to inhibit bradykinin-induced nitric oxide (NO) production in vascular endothelial cells [25], the effect of endostatin on bradykinin-induced myocardial contraction has not been determined. In the present study, we examined the effects of endostatin on basal and bradykinin-induced left atrial contraction and explored underlying mechanisms.

MATERIALS AND METHODS

Reagents: Recombinant mice endostatin, bradykinin and propranolol (Sigma Aldrich, St. Louis, MO, U.S.A.); Hoe140 (Peptide Institute, Osaka, Japan); and Nicardipine (Yamanouchi Pharmaceutical Co., Tokyo, Japan).

Animals: All animal experiments were conducted in accordance with the Guide for Care and Use of laboratory animals as adopted by Kitasato University. Male ddY mice (Japan SLC, Hamamatsu, Japan) and guinea pigs (Japan SLC) were used in this study.

Measurement of isometric contraction: Isometric contraction was measured as described previously [14]. Briefly, left atria of mice (32–55 g body weight) were isolated under pentobarbital anesthesia (50 mg/kg i.p.). Left atrium was placed horizontally in a 10-m*l* tissue bath filled with Krebs-Henseleit solution: 119 mM NaCl, 4.8 mM KCl, 24.9 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$ and 10 mM glucose. The solution was gassed with 95% O_2, 5% CO_2 and maintained at 35.5°C. The atrium was driven by rectangular current pulses via a pair of platinum electrodes (field stimulation, 1 Hz, 5 msec and 1.5 times threshold voltage) connected to an electronic stimulator (ELEC-

TONIC STIMULATOR; Nihon Kohden, Tokyo, Japan) and equilibrated at least for 70 min. Isometric contraction was recorded with a force-displacement transducer (TB-651T; Nihon Kohden) and monitored with a computer-supported data acquisition system (PowerLab; Bioresearch Center, Nagoya, Japan).

Whole cell patch clamp method: Isolation of ventricular myocytes was performed as described previously [16]. Briefly, the heart of adult guinea pigs (220–470 g body weight) was isolated under sodium pentobarbital anesthesia (70 mg/kg, i.p) and perfused by a modified Langendorff apparatus. The perfused heart was digested with 0.02% collagenase, and ventricular myocytes were isolated. Electrophysiological recording of membrane currents was performed by a whole cell patch clamp technique as described previously [16]. The external solution contained the following compositions: 143 mM NaCl, 5.4 mM KCl, 0.33 mM NaH_2PO_4 $2H_2O$, 0.5 mM $MgCl_2$ $6H_2O$, 5.5 mM glucose, 5 mM HEPES and 1.8 mM $CaCl_2$ adjusted to pH 7.4 with NaOH. The temperature of external solution was kept constant at 36°C. Glass patch pipettes were filled with a pipette solution. The pipette solution contained the following compositions: 110 mM KOH, 110 mM l-Aspartate, 20 mM KCl, 1 mM $MgCl_2$ $6H_2O$, 5 mM ATP-K_2, 5 mM phosphocreatine K_2, 10 mM EGTA, 5 mM HEPES-KOH and 1.42 mM $CaCl_2$ adjusted to pH 7.4 with KOH. The resistance of the patch pipette filled with the pipette solution was 2–3 MΩ. The electrode was connected to a patch clamp amplifier (CEZ-2400; Nihon Kohden), and commanded pulses were generated by pCLAMP software (Axon Instrument, Inc., Foster city, CA, U.S.A.). Membrane current was elicited by 300 msec and 8 steps depolarizing pulses of each +10 mV from a holding potential −40 mV. The sampling rate was set to 1 msec. The current density (pA/pF) was calculated by normalizing current amplitude by cellular membrane capacitance.

Statistical Analysis: All data were expressed as mean ± S.E.M. Statistical analysis was performed by using Student's *t*-test (Figs. 2 and 3; between 2 groups) or one-way analysis of variance followed by Dunnett's (Fig. 1) or Bonferroni's post hoc test (Fig. 4) (between more than 3 groups). Values of $P<0.05$ were considered as statistically significant.

RESULTS

Endostatin alone treatment had no influence on left atrial contraction: We first investigated the effects of endostatin alone treatment on left atrial contraction. Endostatin (100–1,000 *n*g/m*l*, 10 min) had no effect on basal atrial contraction (n=7) (Fig. 1).

Endostatin inhibited bradykinin-induced left atrial contraction: We next investigated the effect of endostatin on bradykinin-induced contraction. Bradykinin (1 μM) transiently increased left atrial contraction (Fig. 2A and 2B). We confirmed that Hoe140, a B_2R inhibitor (n=5–6, data not shown), or nicardipine, a L-type calcium channel blocker (n=5, data not shown), suppressed the bradykinin-induced contraction. In contrast, propranolol, a β adrenergic receptor inhibitor, did not suppress the bradykinin-induced contraction (n=6–8, data not shown). Endostatin (300 *n*g/m*l*) pretreatment significantly inhibited the bradykinin-induced contraction (Fig. 2A and 2B, $P<0.05$, n=6–7).

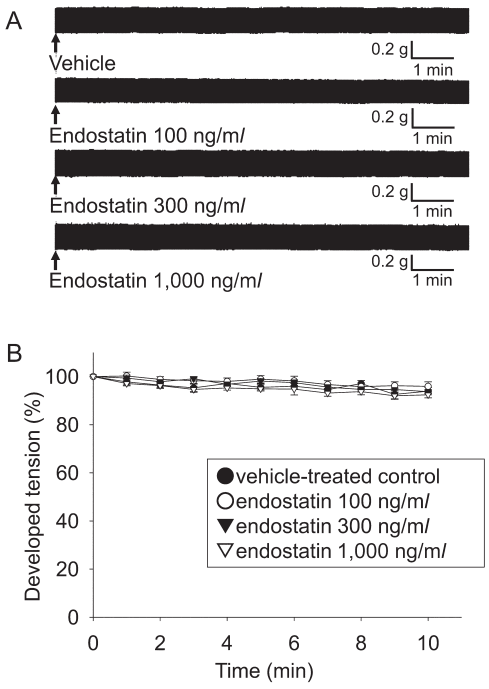

Fig. 1. Endostatin has no influence on basal left atrial contraction. (A) Actual traces for the developed tension in response to vehicle (citric-phosphate buffer) or endostatin in mouse left atrium. Endostatin (100–1,000 *n*g/m*l*) or vehicle was treated (arrows) for 10 min. (B) Time course for the developed tension in response to endostatin or vehicle in the mouse left atrium. The developed tension before endostatin-treatment was set as 100% (n=7).

Endostatin alone treatment had no influence on voltage-dependent calcium current: We next investigated the effects of endostatin alone treatment on voltage-dependent calcium current. Whole-cell patch clamp analysis showed that endostatin alone treatment had no effect on the voltage-dependent calcium current (Fig. 3, n=8).

Endostatin inhibited bradykinin-induced voltage-dependent calcium current: Bradykinin has been reported to increase L-type calcium current in guinea pig cardiomyocytes [19]. We next investigated the effect of endostatin on bradykinin-induced increase of voltage-dependent calcium current. It was confirmed that bradykinin (1 μM) significantly increased voltage-dependent calcium current (Fig. 4, $P<0.01$). Pretreatment with endostatin (300 *n*g/m*l*) significantly inhibited the increased voltage-dependent calcium current induced by bradykinin (Fig. 4, $P<0.01$ at 0 mV, 0.05 at −10 and −20 mV, n=6–11).

DISCUSSION

In the present study, we found that endostatin inhibited bradykinin-induced left atrial contraction. The inhibitory

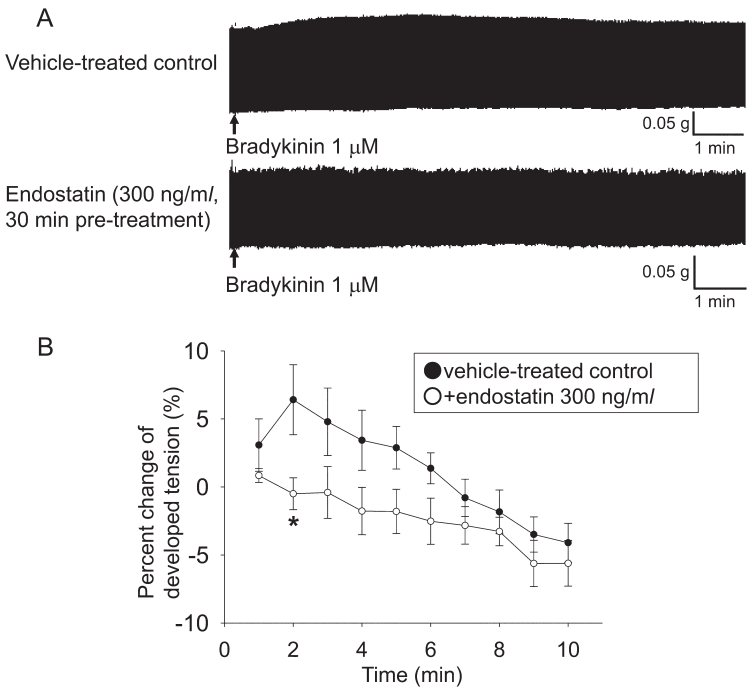

Fig. 2. Endostatin inhibits bradykinin-induced left atrial contraction. (A) Actual traces for the developed tension in response to bradykinin in the mouse left atrium. Endostatin (300 *n*g/m*l*) or vehicle was pre-treated for 30 min before bradykinin (1 μM) treatment (arrows). (B) Time course for the developed tension in response to endostatin in the mouse left atrium. The developed tension before bradykinin-treatment was set as 100%. The changes of the developed tension (1–10 min) were shown (n=6–7). * P<0.05, vs. vehicle-treated control.

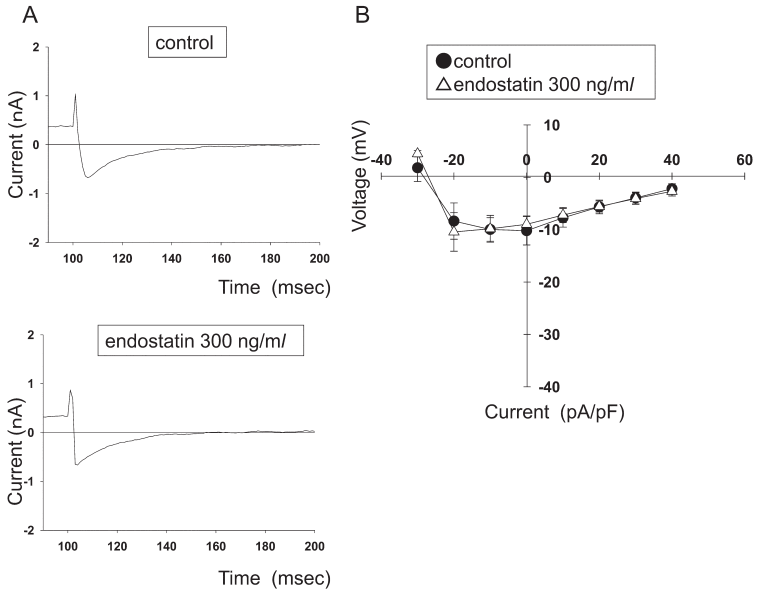

Fig. 3. Endostatin has no influence on voltage-dependent calcium current. Effect of endostatin (300 *n*g/m*l*, 5 min) alone treatment on voltage-dependent calcium current was measured by a whole-cell patch clamp technique in the guinea pig ventricular myocytes. Representative calcium current recording at 0 mV (A, control: upper left, endostatin: lower left) and Current-Voltage relations for the peak of calcium currents (B) were shown. The current density (pA/pF) was calculated by normalizing current amplitude by cellular membrane capacitance. Results were expressed as mean ± S.E.M (n=8).

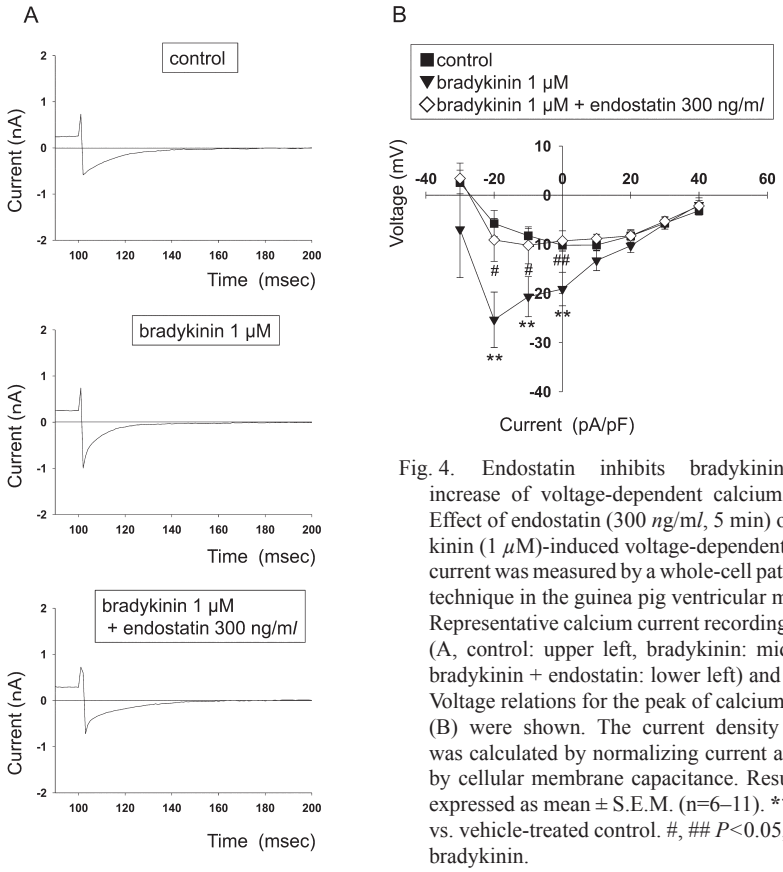

Fig. 4. Endostatin inhibits bradykinin-induced increase of voltage-dependent calcium current. Effect of endostatin (300 *n*g/m*l*, 5 min) on bradykinin (1 μM)-induced voltage-dependent calcium current was measured by a whole-cell patch clamp technique in the guinea pig ventricular myocytes. Representative calcium current recording at 0 mV (A, control: upper left, bradykinin: middle left, bradykinin + endostatin: lower left) and Current-Voltage relations for the peak of calcium currents (B) were shown. The current density (pA/pF) was calculated by normalizing current amplitude by cellular membrane capacitance. Results were expressed as mean ± S.E.M. (n=6–11). ** P<0.01 vs. vehicle-treated control. #, ## P<0.05, 0.01 vs. bradykinin.

mechanism of endostatin might be through the inhibition of voltage-dependent calcium channel. To the best of our knowledge, this study is the first report determining the influence of endostatin on cardiac contraction.

Endostatin is a non-collagenous fragment cleaved from collagen type XVIII [5, 13, 23] and has anti-angiogenic and anti-carcinogenic effects [2–4, 10, 20, 24]. Recently, we have reported that endostatin exerts other roles on cardiac fibroblasts, such as proliferative and migratory roles [15]. Therefore, it is proposed that endostatin may exert various biological effects on cardiac tissues. However, the effect of endostatin on cardiomyocytes, especially cardiac contraction, has not been clarified. We for the first time showed that endostatin alone treatment had no influence on the left atrial contraction. The data that endostatin alone treatment had no influence on voltage-dependent calcium current support it. A report by Zhang *et al.* that endostatin had no effect on L-type calcium current in glioblastoma [26] also supports our data.

Bradykinin is known to increase cardiac contraction via the activation of L-type calcium current through binding to B_2R in guinea pig atria [19]. In the present study, Both Hoe140, a B_2R inhibitor, and nicardipine, a L-type calcium channel inhibitor, suppressed the bradykinin-induced contraction. Tsuda *et al.* reported that bradykinin stimulated noradrenaline release in hypothalamus [21, 22]. In this study, however, propranolol, a β adrenergic receptor inhibitor, did not suppress the bradykinin-induced left atrial contraction. From these results, bradykinin may also enhance left atrial contraction through the increase of L-type calcium current via binding to B_2R in mice.

In the present study, we found that endostatin significantly inhibited bradykinin-induced left atrial contraction. Endostatin also inhibited bradykinin-induced voltage-dependent calcium current. The limitation of this study was that the measurement of contraction was performed in isolated left atria of mouse, while the recording of calcium current was performed in ventricular myocytes of guinea pig (the species different). However, it was previously reported that bradykinin increased cardiac contraction via the activation of L-type calcium current through binding to B_2R in guinea pig isolated atria [19]. It is thus likely that endostatin might inhibit bradykinin-induced contraction in guinea pig isolated cardiac muscle perhaps through the inhibition of L-type calcium channel. Further studies are needed to confirm it by using the tissues and cells from the same species. We previously reported that endostatin stimulated Akt phosphorylation through reactive oxygen species (ROS) production in cardiac fibroblasts [15]. Zhang *et al.* reported that endostatin inhibited bradykinin-induced NO release via ROS production in endothelial cells [25]. Therefore, endostatin might inhibit bradykinin-induced cardiac contraction through the ROS production. Further experiments are needed to clarify this point.

In conclusion, we for the first time demonstrate that endostatin might inhibit bradykinin-induced cardiac contraction perhaps through the inhibition of voltage-dependent calcium channel.

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