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Original Article

Dexmedetomidine-induced contraction involves tyrosine kinase-mediated calcium sensitization in isolated rat aortae

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Abstract: The goal of this study was to investigate the role of tyrosine kinase in contraction induced by the highly selective alpha-2 adrenoceptor agonist dexmedetomidine, which has been widely used for sedation in various procedures in isolated endothelium-denuded rat aortae and the tyrosine kinase-mediated pathway. The effects of genistein, tyrphostin 23, sodium orthovanadate, 1-butanol and 2-butanol on dexmedetomidine-induced contraction were examined. The effect of genistein on the simultaneous intracellular calcium level ($[Ca^{2+}]_i$)-tension curves induced by dexmedetomidine in fura-2-loaded aortic strips was also investigated. Additionally, the effects of rauwolscine and genistein on dexmedetomidine-induced phosphorylation of protein tyrosine, c-Jun NH_2-terminal kinase (JNK), and caldesmon in rat aortic vascular smooth muscle cells were examined using Western blotting. The effects of rauwolscine, genistein, and 1-butanol on dexmedetomidine-induced phospholipase D (PLD) activity in rat aortic vascular smooth muscle cells were also investigated. Genistein, tyrphostin 23 and 1-butanol attenuated the dexmedetomidine-induced contraction whereas sodium orthovanadate enhanced it. Both 1-butanol (0.05%) and its inactive congener 2-butanol (0.05%) attenuated dexmedetomidine (10^{-6} M)-induced contraction. However, 1-butanol attenuated dexmedetomidine (10^{-6} M)-induced contraction to a greater extent compared to 2-butanol. Rauwolscine and genistein attenuated dexmedetomidine-induced phosphorylation of protein tyrosine, JNK, and caldesmon and genistein shifted the slope of the $[Ca^{2+}]_i$-tension curves induced by dexmedetomidine downward. Rauwolscine, genistein, and 1-butanol attenuated dexmedetomidine-induced PLD activity. Taken together, these results suggest that dexmedetomidine-induced contraction involves tyrosine kinase-induced calcium sensitization, which seems to be mediated by either JNK and caldesmon or PLD.

Keywords: Dexmedetomidine, tyrosine kinase, contraction, phospholipase D, calcium sensitization, JNK, caldesmon.

Introduction

The ability of the alpha-2 adrenoceptor agonist dexmedetomidine to induce sedation and analgesia has led to its use in the perioperative period [1]. Intravenous injection of dexmedetomidine causes an initial transient hypertension that is associated with direct stimulation of the alpha-2B adrenoceptor in the vascular smooth muscle before stimulation of the alpha-2 adrenoceptor in the central nervous system to produce the sympatholytic effect [1-6]. In addition, high-dose dexmedetomidine produces severe hypertension [7-9].

Protein tyrosine phosphorylation induced by tyrosine kinase in vascular smooth muscle reportedly causes calcium sensitization through a pathway involving mitogen-activated protein kinase (MAPK) or phospholipase D (PLD), which seems to be associated with the phosphorylation of caldesmon or protein kinase C

(PKC), respectively [10, 11]. Dexmedetomidine-induced contraction is mediated by either c-Jun NH_2-terminal kinase (JNK) phosphorylation via 5-lipoxygenase or caldesmon phosphorylation induced by JNK and PKC [12, 13]. In addition, dexmedetomidine produces calcium sensitization-mediated contraction via Rho-kinase and PKC [14, 15]. Contraction induced by the alpha-2 adrenoceptor agonist UK14304 has been reported to involve tyrosine kinase activation and tyrosine kinase-mediated PLD activation [16, 17]. However, the cellular signaling pathway associated with alpha-2 adrenoceptor-induced tyrosine kinase activation remains unknown. Thus, the goal of this *in vitro* study was to investigate the role of protein tyrosine phosphorylation on the contraction induced by the highly selective alpha-2 adrenoceptor agonist dexmedetomidine in isolated rat aortae and to examine the tyrosine phosphorylation-mediated signaling pathway.

Materials and methods

All experimental procedures and protocols (GNU-130627-R0041) were approved by the Institutional Animal Care and Use Committee at Gyeongsang National University and were performed to comply with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences [18].

Preparation of aortic rings for tension measurement

Isolated rat aortae (N = 42) were prepared for tension measurement as described previously [19]. Male Sprague-Dawley rats (weight: 250 to 300 g) were anesthetized by passing 100% carbon dioxide into the rat's cage. The descending thoracic aorta was dissected from the perivascular fat and connective tissue under a microscope in Krebs solution composed of NaCl (118 mM), $NaHCO_3$ (25 mM), glucose (11 mM), KCl (4.7 mM), $CaCl_2$ (2.4 mM), $MgSO_4$ (1.2 mM), and KH_2PO_4 (1.2 mM). The aorta was then cut into a 2.5-mm segment and suspended in a Grass isometric transducer (FT-03, Grass Instrument, Quincy, MA, USA) under a resting tension of 3.0 g in a 10 mL organ bath at 37°C. The aorta was continuously aerated with 95% oxygen and 5% carbon dioxide to maintain the pH between 7.35 and 7.45. A 3.0 g resting tension was maintained to equilibrate the aortic ring for 2 hours and the Krebs solution in the organ bath was exchanged with fresh Krebs solution every 30 minutes. A 25-gauge needle was inserted into the lumen of the aortic rings and all of the aortic rings were rolled using two 25-gauge needles to remove the aortic endothelium. Phenylephrine (10^{-8} M) was added to the organ bath containing the endothelium-denuded aorta to verify endothelial removal. During the phenylephrine-induced sustained contraction, acetylcholine (10^{-5} M) was added into the organ bath and an aorta with an acetylcholine-induced relaxation from the phenylephrine-induced contraction of less than 15% was regarded as being endothelium denuded in this experiment. After the endothelium-denuded aortic rings with acetylcholine-induced relaxation were washed with fresh Krebs solution, the baseline resting tension was restored. Contraction was then induced by isotonic 60 mM KCl and was assessed and used as a reference value to express the magnitude of contraction induced by dexmedetomidine or KCl. After the endothelium-denuded aortic rings with contraction induced by isotonic 60 mM KCl were washed with fresh Krebs solution and baseline resting tension was recovered, the following experimental protocols were performed. Because dexmedetomidine and sodium orthovanadate produce endothelial nitric oxide, endothelium-denuded aortic rings pretreated with nitric oxide synthase inhibitor N^W-nitro L-arginine methyl ester (L-NAME, 10^{-4} M) were used to rule out the effect of residual endothelium on the dexmedetomidine-induced contraction [20, 21].

Experimental protocols

First, the effects of the tyrosine kinase inhibitors genistein and tyrphostin 23 on the contraction induced by dexmedetomidine in isolated endothelium-denuded rat aortae pretreated with 10^{-4} M L-NAME were assessed. After isolated endothelium-denuded rat aortae were pretreated with genistein (10^{-5} to 10^{-4} M), tyrphostin 23 (10^{-5} to 10^{-4} M) or dimethyl sulfoxide (DMSO, 0.3 and 1%) for 20 minutes, the concentration-response curves induced by the cumulative addition of dexmedetomidine (10^{-9} to 10^{-6} M) were generated in the presence and absence of inhibitor or DMSO. Concentrations of the tyrosine kinase inhibitors (genistein and tyrphostin 23) were chosen based on previous studies [16, 22-24].

Second, the effect of the tyrosine phosphatase inhibitor sodium orthovanadate on the contraction induced by dexmedetomidine or KCl in isolated endothelium-denuded rat aortae pretreated with L-NAME (10^{-4} M) was investigated. After the endothelium-denuded rat aortae were pretreated with sodium orthovanadate (10^{-5} M) for 20 minutes, the concentration-response curves induced by the cumulative addition of dexmedetomidine (10^{-9} to 10^{-6} M) or KCl (10 to 60 mM) were assessed in the presence and absence of sodium orthovanadate (10^{-5} M). The concentration of the tyrosine phosphatase inhibitor (sodium orthovanadate) was chosen on the basis of previous studies [25, 26].

Third, the effect of PLD inhibitor 1-butanol (0.1 to 0.3%) on the contraction induced by dexmedetomidine in isolated endothelium-denuded rat aortae pretreated with L-NAME (10^{-4} M) was investigated. Concentrations of the PLD inhibitor (1-butanol) were chosen based on a previous study [27]. After the endothelium-denuded aortic rings were pretreated with 1-butanol (0.1 to 0.3%) for 20 minutes, the concentration-response curves induced by the cumulative addition of dexmedetomidine (10^{-9} to 10^{-6} M) were generated in the presence and absence of 1-butanol. In addition, the effects of PLD inhibitor 1-butanol (0.05%) and its inactive congener 2-butanol (0.05%) on the dexmedetomidine (10^{-6} M)-induced contraction in endothelium-denuded rat aortae were investigated [27-29]. After dexmedetomidine (10^{-6} M) produced a sustained and stable contraction, 1-butanol (0.05%) or 2-butanol (0.05%) was added.

Fura-2 loading and simultaneous measurements of muscle tension and intracellular calcium level ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured according to the method described by Ok et al. using the fluorescent Ca^{2+} indicator fura-2 [19, 30]. Male Sprague-Dawley rats (body weight: 250-350 g, N = 25) were sacrificed by intraperitoneal administration of sodium thiopental (50 mg/mL) and exsanguination. The descending thoracic aortae were isolated and dissected from perivascular fat and connective tissue under a microscope in Krebs solution. Helically cut muscle strips were exposed to the acetoxymethyl ester of fura-2 (fura-2/AM, 10^{-5} M) in the presence of 0.02% cremophor EL for 5-6 h at room temperature (22-24°C). After fura-2 loading, aortic strips were held horizontally in a 7-mL organ bath (CAF-100; Jasco, Tokyo, Japan) and washed with Krebs solution at 37°C for 20 minutes to remove uncleaved fura-2/AM. Isometric tension was measured with a force-displacement transducer (MLT050, ADInstruments, Colorado Springs, CO, USA) and the muscle strips were alternately illuminated (120 Hz) with 340- and 380-nm lights. The ratios of the 500-nm fluorescence induced by 340-nm excitation (F340) and that induced by 380-nm excitation (F380) were detected with a photomultiplier (CAF-110, Japan Spectroscopic, Tokyo, Japan) and the F340/F380 ratio was used as an indicator of $[Ca^{2+}]_i$. As the dissociation constant of the fluorescent indicator for $[Ca^{2+}]_i$ in cytosol may be different from that *in vivo*, the absolute concentration of $[Ca^{2+}]_i$ was not calculated in the current study [31]. The tension and F340/F380 ratios induced by 60 mM KCl were used as reference values. Isometric tension and the F340/F380 ratio were recorded on a computer equipped with PowerLab/400 and analyzed using the Chart 5 program (ADInstruments). A resting tension of 3.0 g was used in the fura-2-loaded aortic strips. After the aortic strips were pretreated with genistein (10^{-5} M) for 20 minutes, the simultaneous $[Ca^{2+}]_i$-tension curves induced by the cumulative addition of dexmedetomidine were investigated in the presence and absence of genistein.

Cell culture

Rat aortic vascular smooth muscle cells (RAVSMCs) were isolated from descending thoracic aortae following enzymatic dissociation and then cultured in Dulbecco's Modified Eagle's Medium (HyClone, GE Healthcare, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Life technologies, NY, USA) containing 100 U/ml penicillin and 100 μg/ml streptomycin, as previously reported [19]. The cells were plated onto a 100-mm culture dish and incubated at 37°C in 5% CO_2. Upon reaching confluence, the cells were trypsinized (0.05% Trypsin-EDTA) and subcultured in 1:4 ratios. For all further studies, cells between passages 2 and 10 were seeded onto 10-mm dishes (10^7 cells) and cultured until they reached 70% confluence, followed by

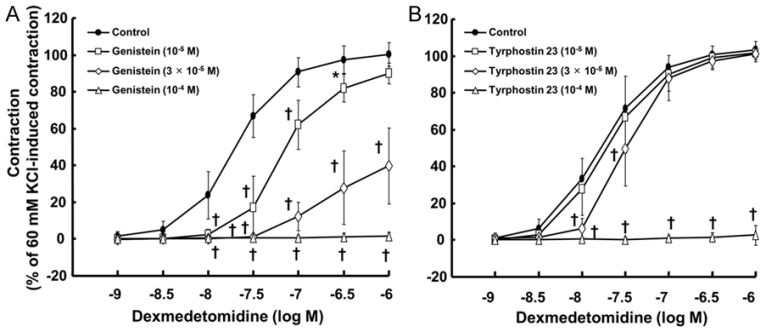

Figure 1. Effect of genistein (A, N = 6) and tyrphostin 23 (B, N = 6) on the contraction induced by dexmedetomidine in isolated endothelium-denuded rat aortae. Data are shown as the mean ± SD and expressed as the percentage of the maximal contraction induced by isotonic 60 mM KCl. N indicates the number of rats from which the descending thoracic aortic rings were derived. $^{*}P < 0.05$ and $^{\dagger}P < 0.001$ versus control. (A) 60 mM KCl-induced contraction: 100% = 1.91 ± 0.29 g, 100% = 1.97 ± 0.32 g, 100% = 1.99 ± 0.30 g and 100% = 1.67 ± 0.34 g for isolated rat aortae pretreated with control, 10^{-5} M genistein, 3×10^{-5} genistein and 10^{-4} M genistein, respectively. (B) 60 mM KCl-induced contraction: 100% = 1.64 ± 0.36 g, 100% = 1.65 ± 0.21 g, 100% = 1.92 ± 0.07 g and 100% = 1.61 ± 0.31 g for isolated rat aortae pretreated with control, 10^{-5} M tyrphostin 23, 3×10^{-5} tyrphostin 23 and 10^{-4} M tyrphostin 23, respectively.

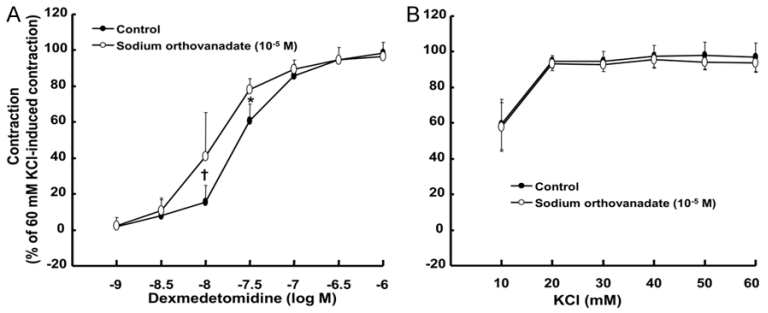

Figure 2. A. Effect of sodium orthovanadate (N = 6) on the contraction induced by dexmedetomidine in isolated endothelium-denuded rat aortae. Data are shown as the mean ± SD and expressed as the percentage of maximal contraction induced by isotonic 60 mM KCl. For 60 mM KCl-induced contraction, 100% = 1.48 ± 0.32 g and 100% = 1.55 ± 0.21 g for isolated rat aortae pretreated with control and 10^{-5} M sodium orthovanadate, respectively. N indicates the number of rats from which the descending thoracic aortic rings were derived. $^{*}P < 0.01$ and $^{\dagger}P < 0.001$ versus control. B. Effect of sodium orthovanadate (N = 10) on the dose-response curve induced by KCl in isolated endothelium-denuded rat aortae. Data are shown as the mean ± SD and expressed as the percentage of maximal contraction induced by isotonic 60 mM KCl. For 60 mM KCl-induced contraction, 100% = 1.65 ± 0.53 g and 100% = 1.92 ± 0.43 g for isolated rat aortae pretreated with control and 10^{-5} M sodium orthovanadate, respectively. N indicates the number of descending thoracic aortic rings.

serum starvation overnight before drug treatment.

Western blot analysis

Western blot analysis was performed following our previously reported protocol [19]. Briefly, proteins were extracted from the cells using RIPA buffer and protein concentrations were determined by Bradford method. A total of 30 μg protein was separated on a 7% or 10% sodium dodecyl sulfate-polyacrylamide gel by electrophoresis for 90 minutes at 100 v. The separated proteins were wet transferred to polyvinylidene difluoride membranes at 190 mA for 1 hour. After transfer, the membranes were blocked with blocking buffer-5% w/v nonfat dried milk in Tris-buffered saline containing Tween-20 (TBST) for 1 hour at room temperature followed by incubation with specific primary antibodies(anti-phospho-tyrosine, anti-phospho-JNK, anti-phospho-caldesmon, anti-JNK, anti-caldesmon, and anti-β-actin) diluted (1:1000) in blocking buffer and incubated at 4°C overnight. After incubation, the membranes were washed 3 times with TBST and incubated with secondary antibodies tagged with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG diluted (1:5000) in blocking buffer for 1 hour at room temperature. The membranes were washed 5 times with TBST and the fluorescence signals were detected using enhanced chemiluminescence (SuperSignal® West Pico Chemiluminescent Substrate; Thermo Scientific, Rockford, IL, USA) and transferred onto an x-ray film (SuperRX-N Fuji Medical X-ray Film, Japan). Signal intensity was measured using densitometry.

PLD activity measurement

The effects of dexmedetomidine and various inhibitors on PLD activity in RAVSMCs were assessed using an Abcam PLD activity assay kit (Cambridge, MA, USA). Briefly, the RAVSMCs (5×10^{-6}) were cultured in a 10 mm dish over-

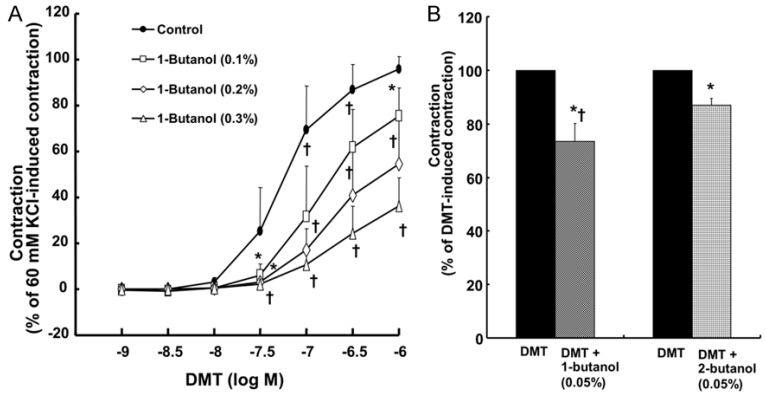

Figure 3. A. Effect of 1-butanol (0.1 to 0.3%; N = 8) on the contraction induced by dexmedetomidine in isolated endothelium-denuded rat aortae. Data are shown as the mean ± SD and expressed as the percentage of contraction induced by isotonic 60 mM KCl. For 60 mM KCl-induced contraction, 100% = 1.60 ± 0.22 g, 100% = 1.72 ± 0.34 g, 100% = 1.67 ± 0.16 g and 100% = 1.75 ± 0.45 g for isolated rat aortae pretreated with control, 0.1% 1-butanol, 0.2% 1-butanol, and 0.3% 1-butanol, respectively. N indicates the number of descending thoracic aortic rings. $^{*}P < 0.01$ and $^{\dagger}P < 0.001$ versus control. B. Effects of 1-butanol (0.05%; N = 7) and 2-butanol (0.05%; N = 7) on the contraction induced by dexmedetomidine (DMT, 10^{-6} M) in isolated endothelium-denuded rat aortae. Data are shown as the mean ± SD and expressed as the percentage of contraction induced by DMT (10^{-6} M). Contraction induced by DMT (10^{-6} M): 100% = 1.98 ± 0.42 g and 100% = 2.01 ± 0.59 g in isolated rat aortae with 1-butanol and 2-butanol, respectively. N indicates the number of descending thoracic aortic rings. $^{*}P < 0.001$ versus DMT alone. $^{\dagger}P < 0.001$ versus DMT + 2-butanol.

night in serum-free media followed by treatment with inhibitors rauwolscine (10^{-5} M), genistein (10^{-4} M), and 1-butanol (0.3%) for 1 hour and dexmedetomidine (10^{-6} M) for 5 minutes or dexmedetomidine (10^{-6} M) alone for 5 minutes. After the treatment, cells were harvested in PLD-assay buffer and PLD activity was measured following the manufacturer's instructions. Absorbance was read at 570 nm on a microplate reader (Versamax, Molecular devices, CA, USA) and optical density from the standard was used to calculate the amount of choline (nmol) generated by PLD from the various treatments. The assay was carried out in four independent experiments.

Materials

All chemicals were commercially available and of the highest purity. Genistein, tyrphostin 23, sodium orthovanadate, 1-butanol, 2-butanol, L-NAME, dexmedetomidine, and rauwolscine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-phospho-JNK (Thr183/Tyr185), anti-phospho-tyrosine, and anti-JNK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-caldesmon and anti-phospho-caldesmon (Ser789) antibodies were purchase from Abcam (Cambridge Science Park, Cambridge, England) and Millipore (Billerica, MA, USA), respectively. Fura-2/AM was purchased from Molecular Probes (Eugene, OR, USA). Genistein and tyrphostin 23 were dissolved in DMSO and all other drugs were dissolved in distilled water. The stock solutions of genistein and tyrphostin 23 dissolved in DMSO were 5 × 10^{-2} and 10^{-2} M, respectively.

Data analysis

The values were expressed as the mean ± SD. Vasoconstriction induced by dexmedetomidine or KCl was expressed as a percentage of the maximal contraction induced by isotonic 60 mM KCl. The effects of various inhibitors and DMSO on the contraction or $[Ca^{2+}]_i$ induced by dexmedetomidine and KCl were analyzed using two-way repeated measures analysis of variance (ANOVA) followed by Bonferroni's post hoc test. The effects of 1-butanol and 2-butanol on the contraction induced by dexmedetomidine (10^{-6} M) were analyzed using an unpaired Student's t-test. The effects of inhibitors on the phosphorylation of protein kinase, JNK, and caldesmon induced by dexmedetomidine and dexmedetomidine-induced PLD activity were analyzed using one-way ANOVA followed by Bonferroni's post hoc test. The slopes of simultaneous $[Ca^{2+}]_i$-tension curves induced by dexmedetomidine in the presence or absence of genistein were calculated using linear regression. The effect of genistein on the slope of simultaneous $[Ca^{2+}]_i$-tension curves evoked by dexmedetomidine was analyzed using an unpaired Student's t-test. *P* values less than 0.05 were considered statistically significant.

Results

Genistein (10^{-5} to 10^{-4} M) and tyrphostin 23 (3 × 10^{-5} and 10^{-4} M) attenuated the dexmedetomidine-induced contraction (**Figure 1A** and **1B**;

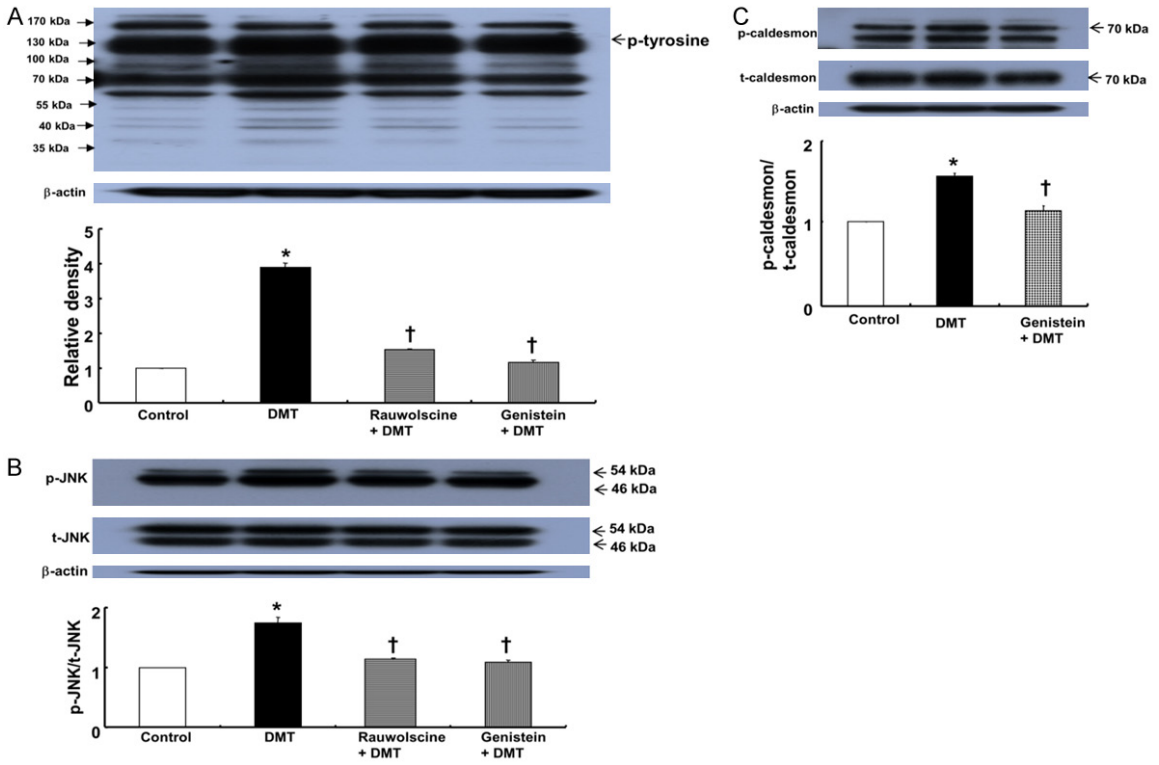

Figure 4. A and B: Effect of rauwolscine (N = 3) and genistein (N = 3) on protein tyrosine and c-Jun NH_2-terminal kinase (JNK) phosphorylation induced by dexmedetomidine (DMT) in rat aortic vascular smooth muscle cells (RAVSMCs). RAVSMCs were treated with 10^{-6} M DMT alone for 10 min or pretreated with 10^{-5} M rauwolscine or 10^{-4} M genistein for 1 h, followed by posttreatment with 10^{-6} M DMT for 10 min. Data are shown as the mean ± SD. N indicates the number of independent experiments. p-tyrosine: phosphorylated protein tyrosine, p-JNK: phosphorylated JNK, t-JNK: total JNK. $^{*}P < 0.001$ versus control. $^{\dagger}P < 0.001$ versus 10^{-6} M DMT. C: Effect of genistein (N = 4) on the caldesmon phosphorylation induced by DMT in RAVSMCs. RAVSMCs were treated with 10^{-6} M DMT alone for 15 min or pretreated with 10^{-4} M genistein for 1 h, followed by posttreatment with 10^{-6} M DMT for 15 min. Data are shown as the mean ± SD. N indicates the number of independent experiments. p-caldesmon: phosphorylated caldesmon, t-caldesmon: total caldesmon. $^{*}P < 0.001$ versus control. $^{\dagger}P < 0.001$ versus 10^{-6} M DMT.

genistein: $P < 0.05$ *vs*. control at 10^{-8} to 3×10^{-7} M dexmedetomidine; tyrphostin 23; $P < 0.001$ *vs*. control at 10^{-8} and 3×10^{-8} M dexmedetomidine). The highest concentration of DMSO (1%), which corresponds to the DMSO concentration contained in the 10^{-4} M tyrphostin 23, slightly attenuated dexmedetomidine-induced contraction ($P < 0.05$ *vs*. control at 3×10^{-8} M dexmedetomidine, Figure S1). However, the highest tested concentration (10^{-4} M) of genistein and tyrphostin 23 nearly abolished the dexmedetomidine (10^{-6} M)-induced maximal contraction (**Figure 1A** and **1B**; $P < 0.001$ *vs*. control). Sodium orthovanadate (10^{-5} M) enhanced the dexmedetomidine-induced contraction (**Figure 2A**; $P < 0.01$ *vs*. control at 10^{-8} and 3×10^{-8} M dexmedetomidine) whereas it had no effect on the KCl-induced contraction (**Figure 2B**). 1-Butanol (0.1 to 0.3%) attenuated the dexmedetomidine-induced contraction (**Figure 3A**; $P < 0.01$ *vs*. control at 3×10^{-8} to 10^{-6} M dexmedetomidine). Both 1-butanol (0.05%) and its inactive congener 2-butanol (0.05%) attenuated dexmedetomidine (10^{-6} M)-induced contraction ($P < 0.001$; **Figure 3B**). However, 1-butanol attenuated dexmedetomidine (10^{-6} M)-induced contraction to a greater extent compared to 2-butanol ($P < 0.001$; **Figure 3B**). Dexmedetomidine (10^{-6} M) induced protein tyrosine phosphorylation in RAVSMCs (**Figure 4A**; $P < 0.001$ *vs*. control). Both rauwolscine (an alpha-2 adrenoceptor inhibitor) and genistein inhibited dexmedetomidine-induced protein tyrosine phosphorylation (**Figure 4A**; $P < 0.001$ *vs*. dexmedetomidine alone). Dexmedetomidine (10^{-6} M) induced JNK phosphorylation in RAVSMCs (**Figure 4B**; $P < 0.001$ *vs*. control) and rauwolscine and genistein inhibited this effect (**Figure 4B**; $P < 0.001$ versus dexmedetomidine alone). Dexmedetomidine (10^{-6} M) induced caldesmon phosphorylation in RAVSMCs (**Figure 4C**; $P < 0.001$ *vs*. control) and genistein inhibited this

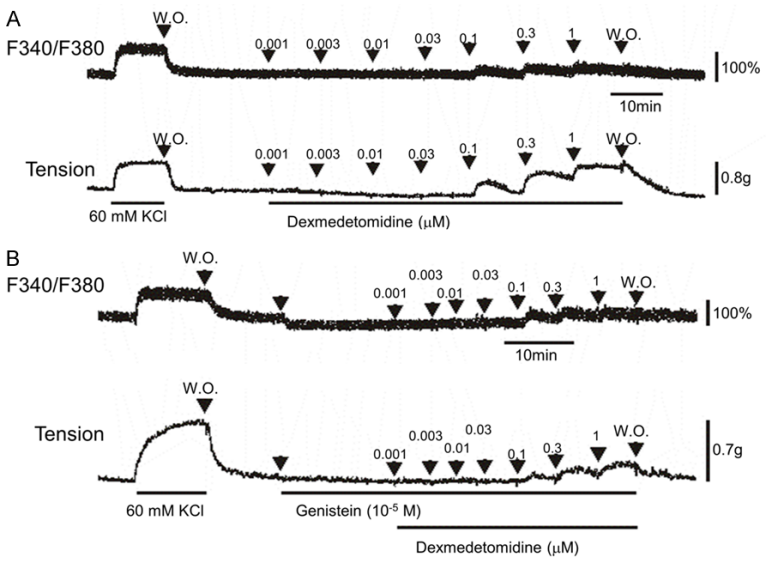

Figure 5. Representative tracing showing the effects of genistein (N = 5) on the intracellular calcium level ($[Ca^{2+}]_i$, upper trace) and tension (lower trace) induced by the cumulative addition of dexmedetomidine in fura-2-loaded aortic strips pretreated with (B) or without (A) genistein. The $[Ca^{2+}]_i$ of fura-2-loaded aortic strips was detected using a fluorometer and expressed as the F340/F380 ratio. After the 60 mM KCl-induced contraction was determined, the aortic strip was washed with fresh Krebs solution, and baseline tension was recovered. Then, dexmedetomidine (10^{-9} to 10^{-6} M) was cumulatively added. N indicates the number of independent experiments. W.O.: washout with fresh Krebs solution.

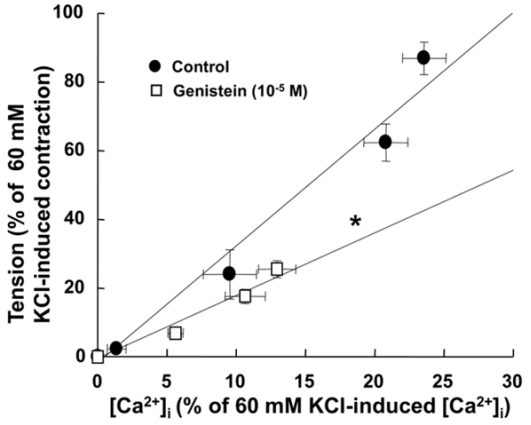

Figure 6. The intracellular calcium level ($[Ca^{2+}]_i$) and tension relationship induced by the cumulative addition of dexmedetomidine (10^{-8} to 10^{-6} M) in the fura-2-loaded aortic strips in the absence and presence of genistein. The $[Ca^{2+}]_i$ and tension induced by dexmedetomidine is expressed as the percentage of $[Ca^{2+}]_i$ and contraction induced by 60 mM KCl, respectively. Data (N = 5) are shown as the mean ± SD. N indicates the number of independent experiments. Slope: $^*P < 0.01$ versus control.

dexmedetomidine-induced caldesmon phosphorylation (**Figure 4C**; $P < 0.001$ *vs.* dexmedetomidine alone).

Dexmedetomidine (10^{-7} to 10^{-6} M) induced a greater contraction than the increase in $[Ca^{2+}]_i$ ($P < 0.05$; **Figure 5A**) whereas pretreatment with genistein (10^{-5} M) abolished the lower dexmedetomidine (10^{-7} M) concentration-induced enhanced contraction but not the dexmedetomidine (10^{-7} M)-induced $[Ca^{2+}]_i$ increase (**Figure 5B**; $P < 0.001$ *vs.* $[Ca^{2+}]_i$ at 3×10^{-7} and 10^{-6} M dexmedetomidine). This suggests that inhibition of the dexmedetomidine-induced contraction by genistein is higher than the inhibition of the dexmedetomidine-induced $[Ca^{2+}]_i$ increase by genistein. Genistein significantly decreased the slope of the $[Ca^{2+}]_i$-tension curve induced by dexmedetomidine (**Figure 6**; $P < 0.01$; slope: control = 3.15 ± 0.80 *vs.* genistein = 1.89 ± 0.41).

Dexmedetomidine (10^{-6} M) increased PLD activity (**Figure 7**; $P < 0.001$ *vs.* control) whereas rauwolscine (10^{-5} M), genistein (10^{-4} M), and 1-butanol (0.3%) attenuated the enhanced PLD activity induced by 10^{-6} M dexmedetomidine (**Figure 7**; $P < 0.001$ *vs.* dexmedetomidine alone).

Discussion

This is the first study to suggest that dexmedetomidine-induced contraction involves protein tyrosine phosphorylation-induced calcium sensitization, seeming to be mediated by either JNK and caldesmon or PLD (**Figure 8**). The major findings of this study are as follows: (1) Genistein and tyrphostin 23 attenuated dexmedetomidine-induced contraction whereas sodium orthovanadate enhances it; (2) Genistein and rauwolscine attenuated the phosphorylation of protein tyrosine kinase, JNK, and caldesmon induced by dexmedetomidine; (3) The slope of the dexmedetomidine-induced $[Ca^{2+}]_i$-tension curve was shifted downwards by genistein; and (4) Rauwolscine, genistein, and 1-butanol attenuated dexmedetomidine-induced PLD activity.

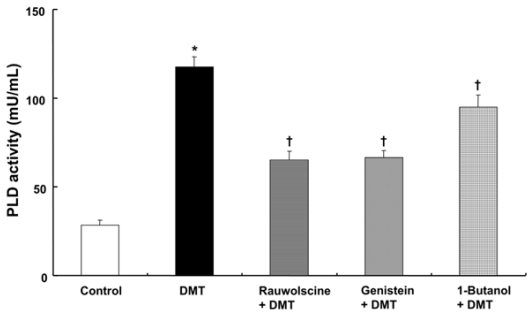

Figure 7. Effect of various inhibitors (10^{-5} M rauwolscine, 10^{-4} M genistein, and 0.3% 1-butanol) on the dexmedetomidine (10^{-6} M, DMT)-induced phospholipase D (PLD) activity in rat aortic vascular smooth muscle cells (RAVSMCs). RAVSMCs were treated with 10^{-6} M DMT alone for 5 min or pretreated with inhibitors (rauwolscine, genistein, and 1-butanol) for 1 h, followed by posttreatment with 10^{-6} M DMT for 5 min. PLD activity was measured as described in the methods. Data (N = 4) are shown as the mean ± SD. N indicates the number of independent experiments. $^{*}P < 0.001$ versus control. $^{\dagger}P < 0.001$ versus DMT alone.

Several contractile agonists including endothelin, serotonin, angiotensin II, and phenylephrine induce contraction mediated by activation of tyrosine kinase [32, 33]. The contraction induced by alpha-2 adrenoceptor agonist UK 14304 is reportedly mediated by the activation of tyrosine kinase [16]. Similar to a previous report, tyrosine kinase inhibitors genistein and tyrphostin 23 inhibited the contraction induced by dexmedetomidine in the current study whereas the tyrosine phosphatase inhibitor sodium orthovanadate increased this contraction [16]. Although DMSO (1%), which is equivalent to the amount of DMSO contained in 10^{-4} M tyrphostin 23, slightly attenuated the dexmedetomidine-induced contraction (Figure S1), as 10^{-4} M tyrphostin 23 nearly abolished the dexmedetomidine-induced contraction (**Figure 1B**), the tyrphostin 23 (10^{-4} M)-mediated inhibition of dexmedetomidine-induced contraction could be ascribed to the inhibition of tyrosine kinase. The current and previous results suggest that dexmedetomidine-induced contraction is mediated by tyrosine kinase activation [16]. Genistein reportedly has no effect on high-KCl-induced contraction [16]. Similarly, sodium orthovanadate had no effect on high-KCl-induced contraction in the current study, suggesting that neither genistein nor sodium orthovanadate (10^{-5} M) has an effect on voltage-operated calcium channel-induced contraction [16]. The PLD inhibitor 1-butanol, which inhibits PLD-induced hydrolyzation of phosphatidylcholine into diacylglycerol and then activates PKC, which is involved in calcium sensitization, attenuated dexmedetomidine-induced contraction [11]. As alcohol has non-specific actions independent of PLD inhibition, we compared the effects of 1-butanol and its inactive congener 2-butanol on the dexmedetomidine-induced contraction [34]. As the dexmedetomidine-induced contraction was more strongly attenuated by 1-butanol than by 2-butanol (**Figure 3B**) in the current study and 1-butanol was reported to inhibit PLD, 1-butanol-mediated inhibition seems to be associated with the inhibition of PLD involved in dexmedetomidine-induced contraction [27-29]. Moreover, the contraction induced by the alpha-2 adrenoceptor agonist UK 14304 involves tyrosine kinase-mediated PLD activation [17]. Reportedly, 1-butanol has no effect on high-KCl-induced contraction and rauwolscine, genistein. 1-butanol attenuated the dexmedetomidine-induced PLD activity in the current study, suggesting that contraction induced by dexmedetomidine is mediated by the activation of PLD by tyrosine kinase via the alpha-2 adrenoceptor, in agreement with previous studies [17]. PLD is involved in the regulation of cellular physiology including membrane trafficking, cytoskeletal reorganization, and receptor-mediated response [35]. Two isoforms of PLD, PLD1 and PLD2 are expressed in mammalian cells [35]. Thus, further study regarding the PLD isoform induced by dexmedetomidine is needed. Tyrosine kinase reportedly activates PLD-induced PKC which leads to calcium sensitization [10, 11]. In addition, dexmedetomidine-evoked JNK phosphorylation has been reported to be induced by PKC-delta [36]. Thus, further study regarding the cellular signaling pathways downstream of dexmedetomidine-induced PLD activation is needed.

Consistent with the tyrosine kinase inhibitor-mediated inhibition of contraction induced by dexmedetomidine obtained from the current isometric tension study, genistein attenuated protein tyrosine phosphorylation induced by dexmedetomidine. In agreement with previous reports that dexmedetomidine-induced contraction is mediated by JNK phosphorylation, dexmedetomidine induced JNK phosphorylation, which was attenuated by genistein [12, 13, 36, 37]. Additionally, the alpha-2 adrenoceptor inhibitor rauwolscine attenuated phosphorylation of protein tyrosine and JNK induced by dexmedetomidine. Taken together with

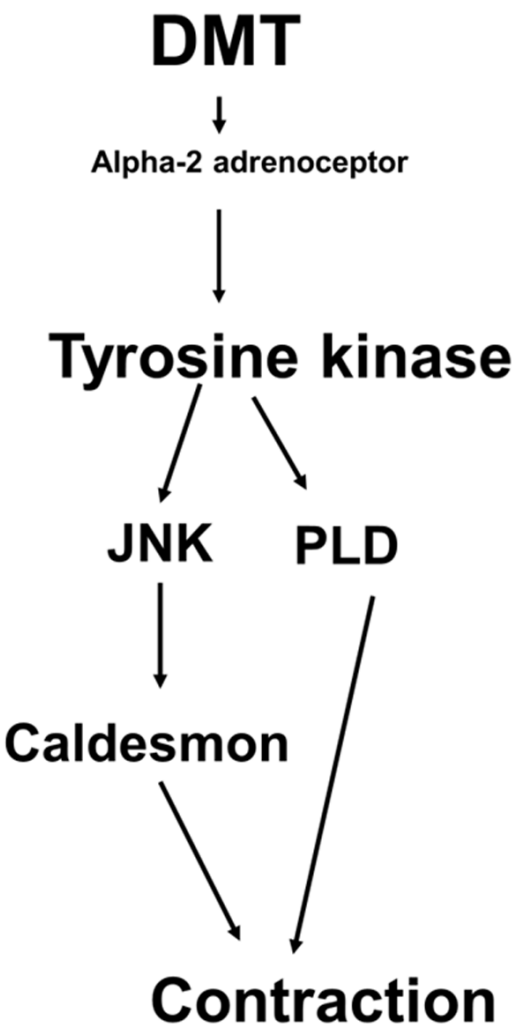

Figure 8. The presumed cellular signaling pathway associated with tyrosine kinase-mediated dexmedetomidine (DMT)-induced contraction in isolated endothelium-denuded rat aortae. JNK: c-Jun NH_2-terminal kinase. PLD: phospholipase D [12].

the results of a previous report, these results suggest that the pathway involving JNK phosphorylation induced by tyrosine kinase activated by the alpha-2 adrenoceptor contributes to dexmedetomidine-induced contraction [37]. Dexmedetomidine-induced phosphorylation of caldesmon, which is an inhibitory actin-binding protein that attenuates the actin-myosin interaction, is reportedly mediated by JNK phosphorylation in RAVSMCs [12, 38]. Taken together with the current results and previous reports, the phosphorylation of caldesmon observed in this study seems to be mediated by a pathway involving the alpha-2 adrenoceptor, tyrosine kinase, and JNK (**Figure 8**) [12, 38]. Norepinephrine activates PLD through an extracellular signal-regulated kinase via tyrosine phosphorylation [39]. Furthermore, the PLD inhibitor 1-butanol attenuated the contraction induced by dexmedetomidine, suggesting that further research regarding the relationship among the contributions of PLD, PKC and JNK to dexmedetomidine-induced contraction is needed [14, 37].

Calcium sensitization induced by dexmedetomidine is mediated by the activation of the phosphorylation-dependent inhibitory protein myosin phosphatase by Rho-kinase and PKC [15]. Genistein, reportedly, attenuates norepinephrine-induced contraction without affecting the increase in $[Ca^{2+}]_i$ induced by norepinephrine [40]. Because tyrosine kinase reportedly activates PLD-induced PKC or MAPK-induced caldesmon phosphorylation, the calcium sensitization-mediated contraction induced by tyrosine kinase seems to be mediated by PLD and MAPK [10, 11, 38]. The genistein-mediated inhibition of dexmedetomidine-induced contraction in our current study was greater than the inhibition of the dexmedetomidine-induced $[Ca^{2+}]_i$ increase. Considering the previous reports and the current results showing that genistein caused a downward shift in the slope of the $[Ca^{2+}]_i$-tension curve induced by dexmedetomidine and attenuated dexmedetomidine-induced PLD activity and phosphorylation of protein tyrosine, JNK, and caldesmon, cellular signaling pathways involving either the alpha-2 adrenoceptor, tyrosine kinase, JNK, and caldesmon or the alpha-2 adrenoceptor, tyrosine kinase, and PLD appear to contribute to dexmedetomidine-induced calcium sensitization (**Figure 8**) [10, 12, 17].

The limitations of this study are as follows. First, endothelial nitric oxide release induced by dexmedetomidine has been reported to attenuate dexmedetomidine-induced contraction [20]. Thus, vasoconstriction induced by a high dose of dexmedetomidine observed in the current study would be attenuated in an *in vivo* state compared with what is observed in the *in vitro* state used in this experiment. Second, blood pressure is mainly affected by small-resistance arterioles but aortae, which are considered to be conduit vessels, were used in this study [41]. Third, simultaneous $[Ca^{2+}]_i$-tension measurements were performed using rat aortic strips whereas data from the biochemical study were obtained from cultured smooth muscle cells. This discrepancy may have affected our present study. However, despite these limita-

tions, dexmedetomidine-induced contraction mediated by tyrosine phosphorylation-induced calcium sensitization may contribute to the hypertension observed in previous studies [2-4, 7-9].

In conclusion, these results suggest that dexmedetomidine-induced contraction involves protein tyrosine phosphorylation associated with calcium sensitization which seems to be mediated by downstream cellular signaling pathways involving either JNK and caldesmon phosphorylation or PLD activation (**Figure 8**).

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Disclosure of conflict of interest

None.

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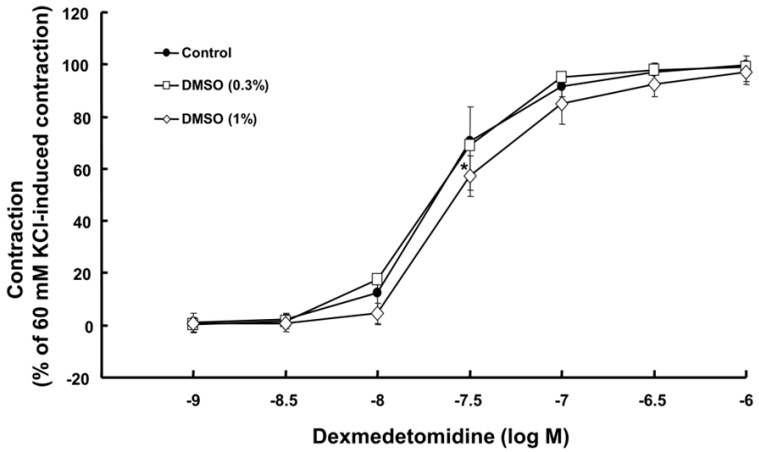

Figure S1. Effect of dimethyl sulfoxide (DMSO; N = 7) on the contraction induced by dexmedetomidine in isolated endothelium-denuded rat aortae. Data are shown as the mean ± SD and expressed as the percentage of the maximal contraction induced by isotonic 60 mM KCl. For 60 mM KCl-induced contraction, 100% = 1.74 ± 0.59 g, 100% = 1.79 ± 0.38 g, and 100% = 1.83 ± 0.46 g for isolated rat aortae pretreated with control, 0.3% DMSO, and 1% DMSO, respectively. N indicates the number of descending thoracic aortic rings. *$P < 0.05$ versus control.