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The Role of Tyrosine Kinase in Ca^{2+}-Independent Contraction of the Ropivacaine on Rat Aortic Smooth Muscle

Zhen Lei[1, *], Jianbo Wu[1, *], Tao Meng[1], Qiyu Bo[1], Xinyu Zhang[1], Jingya Yu[2], and Jingui Yu[1]

[1]*Department of Anesthesiology, Qilu Hospital, Shandong University,107, Wenhua Xi Road, and*
[2]*Institute of Pathogenic Biology, Shandong University School of Medicine, 44, Wenhua Xi Road, Jinan 250012, Shandong, People's Republic of China*

Abstract

The tyrosine kinase signaling pathway plays an important role in the mediation of Ca^{2+} independent mechanisms of smooth muscle contraction. Several components of this pathway, including protein kinase C (PKC), p44/42 mitogen-activated protein kinase (p44/42 MAPK) and Rho-kinase are involved in Ca^{2+} independent mechanisms. Whether the tyrosine kinase pathway mediates vasoconstriction induced by the anesthetic ropivacaine remains unclear. The present study was designed to examine the role of tyrosine kinase in ropivacaine-induced, Ca^{2+}-independent contraction of rat aortic smooth muscle. The effects of tyrosine kinase inhibitor on ropivacaine-induced contractile response were observed by isometric force measurement. The protein tyrosine phosphorylation, PKC, p44/42 MAPK, and membrane translocation of Rho-kinase were examined by Western blotting. Ropivacaine induced a concentration-dependent contractile response, and showed a number of effects on protein tyrosine phosphorylation. In this study, phosphorylation levels were shown to increase at lower concentrations of ropivacaine, but the levels decline at higher concentrations in rat aortic rings attenuated by the tyrosine kinase inhibitor genistein in a concentration-dependent fashion. Ropivacaine-induced phosphorylation of PKC and p44/42 MAPK and Rho-kinase membrane translocation were also significantly attenuated by genistein in similar decreasing manner as the PKC inhibitor bisindolylmaleimide I (Bis I) and the Rho-kinase inhibitor, Y27632, but to a lesser degree than that by the p44/42 MAPK inhibitor, PD 098059. Our results showed that the ropivacaine-induced, Ca^{2+} independent-mediated contraction of rat aortic smooth muscle is, in part, regulated by tyrosine kinase-catalyzed protein tyrosine phosphorylation.

Key Words: aorta, muscle, ropivacaine, smooth, tyrosine kinase

Introduction

It has been well documented that Ca^{2+} independent mechanisms mediate vascular smooth muscle contraction (13), and that the protein kinase C (PKC) (33), p44/42 mitogen-activated protein kinase (p44/42 MAPK) (30), and Rho kinase (9) signalling pathways primarily contribute to Ca^{2+} independent mechanisms. This pathway involves the downregulation of myosin light-chain phosphatase resulting in net increased phosphorylation of myosin, leading to contraction (27). Protein phosphorylation is central importance to excitation-contraction coupling in smooth muscle. Phosphorylation occurs at the alkyl alcohol groups of serine and threonine residues in the target molecules, or at the phenol group of tyrosine

Corresponding author: Jingui Yu, M.D., Ph.D., Department of Anesthesiology, Qilu Hospital, Shandong University, 107, Wenhua Xi Rd., Jinan 250012, Shandong, People's Republic of China. Fax: 86053186073074, E-mail address: yujingui1109@126.com
*These two authors contributed equally to this study.
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targeted by tyrosine kinases. Relatively less is known about tyrosine kinases than about Ser/Thr kinases. Evidence suggests that tyrosine kinase-catalyzed tyrosine phosphorylation plays an important role in mediating vascular contraction via potentiating the activation of PKC, Rho kinase and p44/42 MAPK (4, 5, 32).

Ropivacaine, a new long-acting local anesthetic, is characterized by potent vasoconstriction (12, 15, 18), which likely contributes to its long-acting effects. In a previous study, we have demonstrated that Ca^{2+} independent mechanisms, including PKC, Rho kinase and p44/42 MAPK, are involved in concentration-dependent ropivacaine-induced vascular contraction (39). However, the role of tyrosine kinase in mediating these mechanisms remains unclear.

Genistein is a well established and effective nonselective tyrosine kinase inhibitor (1) which exhibits broad selectivity against tyrosine kinases such as $pp60^{V\text{-}SRC}$, but poorly inhibits serine/threonine kinases such as PKA and PKC (1, 3). Therefore, it is useful as a first step towards probing the tyrosine kinase pathway. This study is designed to investigate the effects of genistein on ropivacaine-induced, Ca^{2+} independent vasoconstriction of rat aortic smooth muscle.

Materials and Methods

Materials

Ropivacaine was a kind gift from AstraZeneca (Shanghai, China). Genistein, Bis I and PD 098059 were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). Y27632 was provided by the Calbiochem-Novabiochem Corporation (La Jolla, CA, USA). The phospho-tyrosine monoclonal antibody (p-Tyr-100), and polyclonal antibodies against phospho-PKC (pan, βIISer660), p44/42 MAPK and phospho-p44/42MAPK (Thr/Tyr204) were supplied by Cell Signaling Technology Inc. (Beverly, MA, USA). Polyclonal antibodies against PKC (H-300) and Rock-2, and the secondary antibody labeled with horseradish peroxidase were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other reagents for the experiments were of analytical grade.

Isometric Tension Measurement

This study was approved by the Animal Care and Use Committee of the institute. Male Wistar rats (300-400 g) were anesthetized with sodium pentobarbital (50 mg/kg) (28) and were exsanguinated by bleeding from the common carotid artery. Endothelium-denuded rat aortic rings were prepared as previously described (10, 31, 34, 37, 38, 40). The rings were placed in 10 ml organ chambers containing Krebs bicarbonate solution of the following composition: 118.2 mM NaCl, 4.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24.8 mM $NaHCO_3$ and 10 mM dextrose. The solution was maintained at pH 7.4 at 37°C under 95% O_2 and 5% CO_2. The cyclooxygenase inhibitor indomethacin (1×10^{-5} M) and the nitric oxide synthase inhibitor N-nitro-L-arginine methyl ester (1×10^{-4} M), were added into the solution to prevent the release of endogenous prostaglandin I_2 and nitric oxide, respectively, from any potentially remaining residual endothelium. The rings were then mounted on a force-displacement transducer (JH-2B, Institute of Space Medical-Engineering, Beijing, PRC) and equilibrated for 1 h at a resting tension of 3 g. The overall contractile responsiveness of the rings was assessed with KCl (3×10^{-2} M) and removal of the endothelium was confirmed with acetylcholine (1×10^{-5} M).

The cumulative concentration-response relationships of the rings to ropivacaine (3×10^{-5} M to 3×10^{-3} M) were examined in the presence or absence of the tyrosine kinase inhibitor genistein in a random manner (5×10^{-6} M and 1×10^{-5} M, 5×10^{-5} M). The effects of the PKC inhibitor Bis I, the p44/42 MAPK inhibitor PD 098059, or the Rho kinase inhibitor Y27632 on ropivacaine-induced contraction were also examined. The rings were incubated with 5×10^{-6} M Bis I, 5×10^{-5} M PD 098059, or 10^{-6} M Y27632 for 15 min before treatment with 3×10^{-4} M ropivacaine. One ring from each animal was randomly challenged by one inhibitor, followed by cumulative treatment with ropivacaine and genistein, or with a single concentration of ropivacaine (3×10^{-4} M) in conjunction with the other inhibitors. The sample size was the number of rings obtained from the same number of rats for each concentration of the inhibitor. Ropivacaine-induced contractile responses were expressed as a percentage of KCl (3×10^{-2} M)-induced contraction.

Detection of Protein Kinase Activation

Endothelium-denuded rat aortic strips (about 3.5 cm in length) were randomly treated with different concentrations of ropivacaine (3×10^{-5} M, 10^{-4} M, 3×10^{-4} M, 1×10^{-3} M or 3×10^{-3} M) for 20 min each to examine the concentration-response relationship of ropivacaine-induced protein tyrosine phosphorylation. Some strips were randomly pretreated with the tyrosine kinase inhibitor genistein (5×10^{-5} M), the PKC inhibitor Bis I (1×10^{-5} M), the p44/42 MAPK inhibitor PD 098059 (1×10^{-4} M), or the Rho-kinase inhibitor Y27632 (5×10^{-6} M) for 15 min. All strips

were then challenged with ropivacaine (3×10^{-4} M) for 20 min to test its inhibitory effects against protein tyrosine phosphorylation, phosphorylation of PKC and p44/42 MAPK, and Rho kinase membrane translocation. Each animal provided only one strip, and each strip was randomly treated with only one concentration of ropivacaine or one kind of inhibitor once.

The agent-treated aortic strips were quickly frozen with dry ice and homogenized in lysis buffer (50 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 50 mM sodium fluoride, 5 mM EDTA, 10 mM sodium pyrophosphate, 1 mM phenylmethanesulfonyl fluoride, 1 mM Na_3VO_4, 10 g/ml leupeptin and 20 g/ml aprotinin) (24). Homogenates were centrifuged at 15,000 *g* for 15 min at 4°C, and the supernatant was isolated for the detection of protein tyrosine, PKC and p44/42MAPKs phosphorylation. For the measurement of Rho-kinase (Rock-2) membrane translocation, the homogenates were centrifuged at 13,000 g for 3 min at 4°C. The supernatant was collected and then centrifuged at 100,000 *g* for 30 min at 4°C. The resultant supernatant (cytosolic fraction) was removed and the pellet (membrane fraction) was resuspended using the same buffer. The protein concentration of each sample was determined using the bicinchoninic acid method (26).

An equivalent amount of total protein (20-30 g) was used for each sample in every experiment. Proteins were separated by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis and were transferred to a nitrocellulose membrane. The membrane was treated with an anti-phospho-tyrosine monoclonal antibody (p-Tyr-100, 1:2000), or anti-PKC (1:1000), anti-phospho-PKC (pan, βIISer660, 1:1000), anti-p44/42 MAPK (1:2000), anti-phospho-p44/42 MAPK (Thr/Tyr204, 1:2000) or anti-Rock-2 (1:1000) antibodies as appropriate for 2 h, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (1:2000) for 1 h at room temperature. Immunoreactive bands were detected using Enhanced Chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK) and were assessed with image analysis software (NIH Image 1.62, National Institutes of Health, Bethesda, MD, USA). The total density of the tyrosine kinase-phosphorylated protein bands was expressed relative to control. The phosphorylation of PKC and p44/42 MAPK was expressed as percentage of the density of total PKC and total p44/42 MAPK bands, respectively. The amount of Rock-2 in the membrane fraction was expressed as a percent of the total Rock-2 value, *i.e.*, membrane fraction plus cytosolic fraction. The sample size represents the number of aortic strips from the same number of rats for each concentration of ropivacaine or each inhibitor.

Statistical Analyses

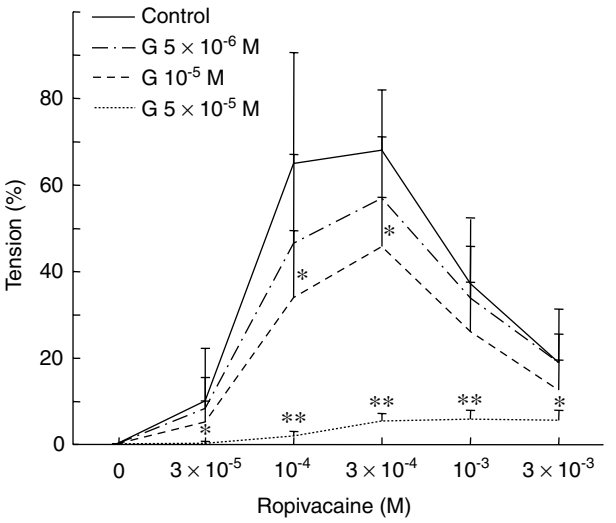

Fig. 1. Effects of the tyrosine kinase inhibitor genistein on ropivacaine-induced, cumulative concentration-dependent contraction. The tension of endothelium-denuded rings was measured using isometric force measurements. Genistein was delivered 15 min prior to the application of ropivacaine. The tension was expressed as a percentage of the KCl (3×10^{-2} M)-induced contraction. $*P < 0.05$ and $**P < 0.01$ *vs*. the control. n = 6. G: genistein.

All data are presented as mean ± SD. The effects of different concentrations of genistein on ropivacaine-induced contraction at each concentration were tested by a one-way analysis of variance followed by an unpaired Student's *t*-test with a Bonferroni correction. The effects of Bis I, PD 098059 and Y27632 on ropivacaine-induced contraction, and the ropivacaine-induced concentration-dependent changes in protein kinase activation were analyzed by two-way analysis of variance for repeated measures and a paired Student's *t*-test with a Bonferroni correction for *post hoc* comparisons. *P*-values < 0.05 were considered statistically significant.

Results

The involvement of tyrosine kinase in the ropivacaine-induced contractions of the rat aortic smooth muscle is shown in Fig. 1. Ropivacaine induced a bell curved, concentration-dependent contraction of endothelium-denuded rat aortic rings at increasing drug concentrations from 3×10^{-5} M to 3×10^{-4} M, and the contraction declined from 10^{-3} M to 3×10^{-3} M drug concentrations, such effects were attenuated by the tyrosine kinase inhibitor genistein in a concentration-dependent manner.

The ropivacaine-induced, tyrosine kinase-mediated protein kinase phosphorylation of the rat aortic smooth muscle is presented in Fig. 2. A set of tyrosine kinase-phosphorylated protein bands

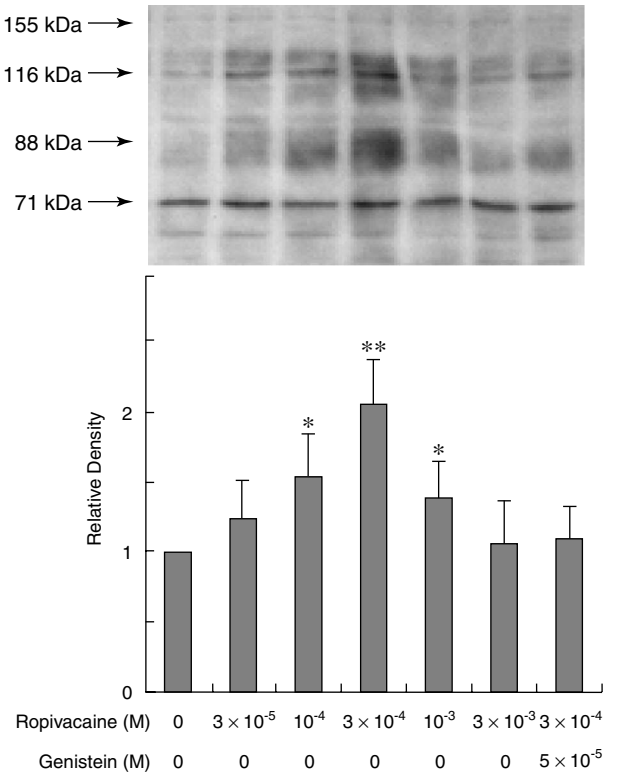

Fig. 2. Ropivacaine-induced protein tyrosine phosphorylation of rat aortic smooth muscle. Endothelium-denuded strips were randomly challenged with ropivacaine for 20 min, or were first pre-treated with genistein (5×10^{-5} M) for 15 min, and then challenged with ropivacaine (3×10^{-4} M) for 20 min. Protein tyrosine phosphorylation was detected using Western blotting with a specific antibody. The total densities of tyrosine phosphorylated bands were expressed relative to controls. $*P < 0.05$ and $**P < 0.01$ *vs*. control. n = 4.

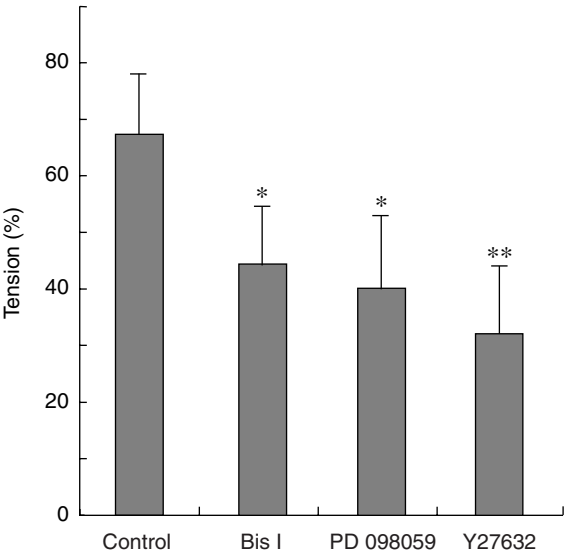

Fig. 3. Effects of Ca^{2+} independent signal pathways inhibitors (Bis I, PD 098059 or Y27632) on ropivacaine-induced contraction of rat aortic smooth muscle. Endothelium-denuded rings were randomly pretreated with the PKC inhibitor Bis I (5×10^{-6} M), the p44/42 MAPK inhibito PD 098059 (5×10^{-5} M) or the Rho kinase inhibitor Y27632 (10^{-6} M) for 15 min before treatment with ropivacaine (3×10^{-4} M). The tension was measured using isometric force measurements, and was expressed as a percent of KCl (3×10^{-2} M)-induced contraction. $*P < 0.05$ and $**P < 0.01$ *vs*. the control. n = 6. Bis I: bisindolylmaleimide I.

prominent at 71, 88, 116 and 155 kDa were detected following stimulation with ropivacaine. The total band densities reached a peak level at 3×10^{-4} M ropivacaine, and subsequently declined, consistent with contractile response. The ropivacaine (3×10^{-4} M)-induced increase in the total densities of tyrosine kinase-phosphorylated bands was significantly attenuated by genistein (5×10^{-5} M).

The effects of contraction-associated inhibitors in ropivacaine-induced contraction of rat aortic smooth muscle are shown in Fig. 3. The ropivacaine (3×10^{-4} M)-induced contraction of endothelium-denuded rat aortic rings was significantly inhibited by the PKC inhibitor Bis I (1×10^{-6} M), the p44/42 MAPK inhibitor PD 098059 (1×10^{-5} M), and the Rho-kinase inhibitor Y27632 (5×10^{-7} M).

The involvement of tyrosine kinase in the ropivacaine-induced activation of PKC, p44/42 MAPK, and Rho kinase in rat aortic smooth muscle was next investigated (Figs. 4-6). Ropivacaine (3×10^{-4} M) induced phosphorylation of PKC and p44/42 MAPK, and Rho-kinase membrane translocation was significantly attenuated by Bis I (1×10^{-5} M), PD 098059 (1×10^{-4} M) and Y27632 (5×10^{-6} M), respectively. The administration of the tyrosine kinase inhibitor genistein (5×10^{-5} M) also inhibited the ropivacaine-induced phosphorylation of PKC and p44/42 MAPK. Rho-kinase membrane translocation was also inhibited by genistein in the same decreasing fashion as Bis I (1×10^{-5} M) and Y27632 (5×10^{-6} M), but to a lesser degree than PD 098059 (10^{-4} M).

Discussion

The findings of the present study that the tyrosine kinase inhibitor genistein, attenuated both ropivacaine-induced contraction and tyrosine kinase-catalyzed protein tyrosine phosphorylation of rat aortic smooth muscle in a concentration-dependent fashion suggest that tyrosine kinase is involved in ropivacaine-induced vascular contraction. The role of tyrosine kinase-mediated protein tyrosine phosphorylation in smooth muscle contraction has been well reviewed (4, 5, 25). In brief, agonist-activated tyrosine kinase phosphorylates a set of substrates including phospho-

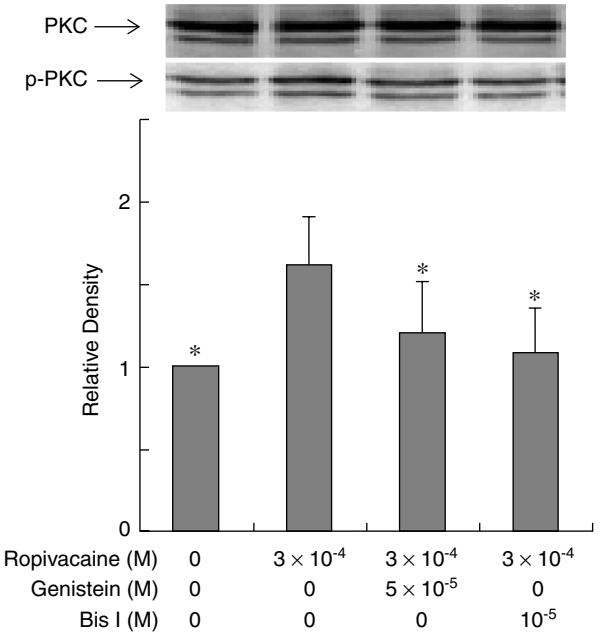

Fig. 4. The role of tyrosine kinase in ropivacaine-induced PKC phosphorylation in rat aortic smooth muscle. Endothelium-denuded strips were treated with ropivacaine (3×10^{-4} M) for 20 min, or initially pre-treated with genistein (5×10^{-5} M) or Bis I (10^{-5} M) for 15 min, and were then challenged with ropivacaine for 20 min. PKC phosphorylation was detected using Western blotting with a specific antibody. The phosphorylation of PKC was expressed as a percentage of the density of total PKC. $^{*}P < 0.05$ *vs*. the value by ropivacaine (3×10^{-4} M), n = 4. There is no difference between the value by Bis I (10^{-5} M) and the value by genistein (5×10^{-5} M) ($P > 0.05$). Bis I: bisindolylmaleimide I; PKC: protein kinase C; p-PKC: phosphorylated protein kinase C.

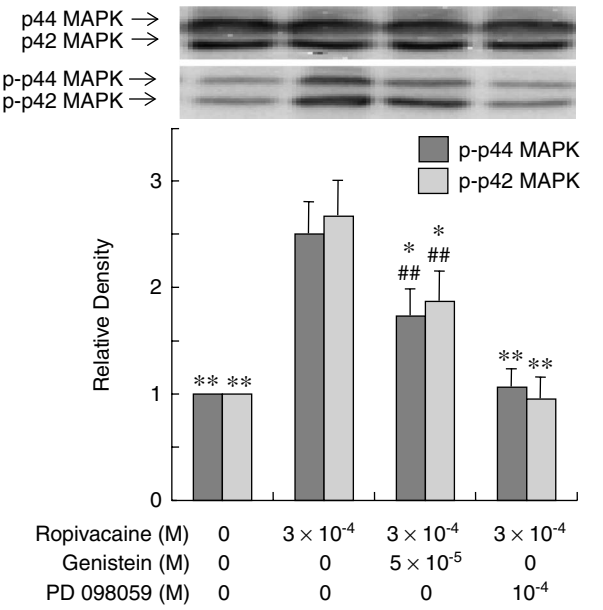

Fig. 5. The role of tyrosine kinase in ropivacaine-induced p44/42 MAPK phosphorylation in rat aortic smooth muscle. Endothelium-denuded strips were treated with ropivacaine (3×10^{-4} M) for 20 min, or first pre-treated with genistein (5×10^{-5} M) or PD 098059 (10^{-4} M) for 15 min, and then challenged with ropivacaine (3×10^{-4} M) for 20 min. p44/42 MAPK phosphorylation was detected using Western blotting with a specific antibody. The phosphorylation of p44/42 MAPK was expressed as a percentage of the total p44/42 MAPK. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. the value by ropivacaine (3×10^{-4} M); $^{\#\#}P < 0.01$ *vs*. the value by PD098059 (10^{-4} M), n = 4. p44/42 MAPK: p44/42 mitogen-activated protein kinase; p-p44/42 MAPK: phosphorylated p44/42 mitogen-activated protein kinase.

lipase γ-1 (21, 23, 32), p120 Ras-GTPase-activating proteins (p120 Ras-GAP) (7) and p190 Rho-GAP (2, 8). Activated phospholipase γ-1 catalyzes the hydrolyzation of phosphotidylinositol 4, 5-bisphosphate to inositol 1, 4, 5 trisphosphate and diacylglycerol. The generation of diacylglycerol activates the PKC/myosin phosphatase pathway (33). Phosphorylated p120 Ras-GAP and p190 Rho-GAP both decrease the dephosphorylation of Ras-GTP and Rho-GTP, stimulating the Ras/Raf/MEK1/2/p44/42 MAPK/caldesmon pathway (30) and the Rho/Rho kinase/myosin phosphatase pathway (9), respectively. Therefore, tyrosine kinase is one of the upstream effectors of these signaling pathways, and can mediate Ca^{2+} independent mechanisms in smooth muscle contraction.

Our findings that genistein inhibited the ropivacaine-elicited phosphorylation of PKC and p44/42 MAPK, and the membrane translocation of Rho kinase could indicate that tyrosine kinase is involved in ropivacaine-induced vascular constriction by mediating Ca^{2+} independent mechanisms. The incomplete inhibition of the phosphorylation of PKC and p44/42 MAPK and membrane translocation of Rho kinase by genistein suggests that, in addition to the tyrosine kinase pathway, some other pathway(s) may also contribute to the mediation of Ca^{2+} independent mechanisms in ropivacaine-induced vasoconstriction. Although the current study demonstrated that ropivacaine stimulated tyrosine kinase activation and contraction of rat aortic smooth muscle in a concentration-dependent manner, the mechanism by which ropivacaine increases and attenuates the activation of tyrosine kinase at low and high concentrations remains unclear.

In our previous study (39), we demonstrated that Ca^{2+} independent mechanisms (including PKC, Rho kinase and p44/42 MAPK) were involved in concentration-dependent ropivacaine-induced vascular contraction. Based on findings of the previous study, our current research further indicated that tyrosine kinase mediated Ca^{2+}-independent contraction through the actions of ropivacaine on rat aortic smooth muscle.

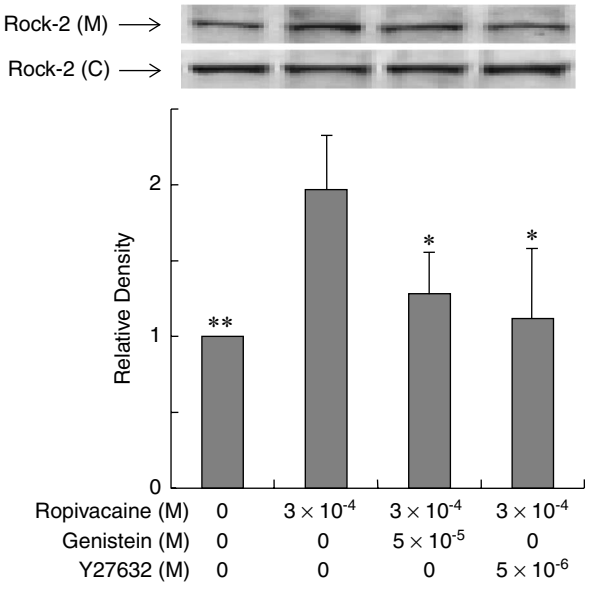

Fig. 6. The role of tyrosine kinase in ropivacaine-induced Rho kinase membrane translocation in rat aortic smooth muscle. Endothelium-denuded strips were treated with ropivacaine (3×10^{-4} M) for 20 min, or first pre-treated with genistein (5×10^{-5} M) or Y 27632 (5×10^{-6} M) for 15 min, and were then challenged with ropivacaine for 20 min. The Rho kinase (Rock-2) was detected on both the membrane and cytosolic fractions using Western blotting with a specific antibody. The level of Rock-2 in the membrane fraction was expressed as a percentage of the total Rock-2, i.e., membrane fraction plus cytosolic fraction. $*P < 0.05$, $**P < 0.01$ *vs*. the value by ropivacaine (3×10^{-4} M). There was no difference between the value by Y27632 (5×10^{-6} M) and the value by genistein (5×10^{-5} M) ($P > 0.05$). n = 4. Y: Y27632; M: membrane fraction; C: cytosolic fraction; Rock-2: one sub-type of Rho kinases.

Protein tyrosine kinases are involved in signal transduction mediated by several vasoagonists and growth factor receptors (14, 17, 21, 35). However, some *in vivo* studies have demonstrated that ropivacaine-mediated contraction of canine spinal and cerebral pial vasculature does not seem to be mediated *via* α- or β-adrenoceptors (15). Other researches have indicated that ropivacaine blocks neuronal norepinephrine reuptake by sympathetic nerve terminals (22) and does not affect ATP-sensitive K^+ channels on isolated rat aortic smooth muscle cells (6). It has also been demonstrated that S-ropivacaine can induce concentration-dependent bell curved contractions in rat aortic smooth muscle through a mechanism that requires extracellular calcium that is mediated by activation of the lipoxygenase pathway (29). Thus, it is unknown what receptors, coupled G proteins or other protein sites through which ropivacaine activates the tyrosine kinase pathway.

In the present study, endothelium-denuded rat aortic strips were used to measure ropivacaine-induced vascular contraction and to study the role of tyrosine kinase in Ca^{2+} independent mechanism-mediated contraction through the actions of ropivacaine, which may be different to that using the intact artery. However, some previous results have indicated that the tyrosine kinase inhibitor genistein completely attenuated the contractions in rat aortic rings induced by most vasoconstrictors regardless of endothelial and non-endothelial functions (16, 20), and removal of the endothelium did not significantly affect the contractile activity of ropivacaine in human mammary artery (11).

In addition to its action as a tyrosine knase inhibitor, genistein also has an estrogen effect *via* binding to the estrogen receptor β, triggering many biological responses (19, 36). Despite this, previous research has suggested that the relaxant effects of vascular contractility induced by genistein are not related to sex hormone receptors (20). Another report demonstrated that phytoestrogen α-zearalanol-induced relaxant effects of rat thoracic aortas rings were also not related with the activation of estrogen receptor (33). We do not rule out the possibility that genistein could interfere with our experiments *via* a tyrosine kinase-independent pathway. However, genistein is a well-used tyrosine knase inhibitor (1, 3). Our study demonstrates that tyrosine kinase-catalyzed protein tyrosine phosphorylation is, at least in part, involved in the ropivacaine-induced, Ca^{2+} independent mechanism-mediated contraction of rat aortic smooth muscle.

Local anesthetics have been widely used in clinical local anesthesia. Because many of these compounds, such as lidocaine, possess little to no vasoconstrictory effects, co-administration of vasoconstrictive compounds is often necessary to decrease absorption by tissues, to extend residence time and to strengthen action of the administered compounds in local and epidural anesthesia. The vasocontractive effects of ropivacaine as demonstrated in our studies suggest that, without adding any vasoconstrictors, local anesthesia or epidural anesthesia using ropivacaine is able to maintain sufficient residential time and stronger action, which is consistent with the clinical situation. Therefore, it is necessary to further investigate the mechanism of ropivacaine-induced vascular contraction. The present study suggests that the mediation of Ca^{2+} independent mechanisms by tyrosine kinases is at least one mechanism through which ropivacaine enhances vascular tension.

Acknowledgments

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