Involvement of the Tyr Kinase/JNK Pathway in Carbachol-induced Bronchial Smooth Muscle Contraction in the Rat

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ABSTRACT

Background: Tyrosine (Tyr) kinases and mitogen-activated protein kinases have been thought to participate in the contractile response in various smooth muscles. The aim of the current study was to investigate the involvement of the Tyr kinase pathway in the contraction of bronchial smooth muscle.

Methods: Ring preparations of bronchi isolated from rats were suspended in an organ bath. Isometric contraction of circular smooth muscle was measured. Immunoblotting was used to examine the phosphorylation of c-Jun N-terminal kinases (JNKs) in bronchial smooth muscle.

Results: To examine the role of mitogen-activated protein kinase(s) in bronchial smooth muscle contraction, the effects of MAPK inhibitors were investigated in this study. The contraction induced by carbachol (CCh) was significantly inhibited by pretreatment with selective Tyr kinase inhibitors (genistein and ST638, n = 6, respectively), and a JNK inhibitor (SP600125, n = 6). The contractions induced by high K+ depolarization (n = 4), orthovanadate (a potent Tyr phosphatase inhibitor) and sodium fluoride (a G protein activator; NaF) were also significantly inhibited by selective Tyr kinase inhibitors and a JNK inhibitor (n = 4, respectively). However, the contraction induced by calcycin-A was not affected by SP600125. On the other hand, JNKs were phosphorylated by CCh (2.2 ± 0.4 [mean±SEM] fold increase). The JNK phosphorylation induced by CCh was significantly inhibited by SP600125 (n = 4).

Conclusion: These findings suggest that the Tyr kinase/JNK pathway may play a role in bronchial smooth muscle contraction. Strategies to inhibit JNK activation may represent a novel therapeutic approach for diseases involving airway obstruction, such as asthma or chronic obstructive pulmonary disease.

AIRWAY hyperresponsiveness and variable airflow obstruction are key features of airway-related diseases such as asthma; indeed, these may be considered the most clinically relevant features of such airway disorders. For this reason, it is essential to have a good understanding of the mechanisms that underlie excitation-contraction coupling in airway smooth muscle.

Recent studies have revealed that agonist-induced smooth muscle contraction is regulated by both cytosolic Ca2+-dependent and Ca2+-independent pathways. The former activates calmodulin/myosin light chain kinase to phosphorylate myosin light chain, while the latter inhibits myosin light chain phosphatase to augment myosin light chain phosphorylation. Agonists such as carbachol (CCh) increase the contractile force further even at a given cytosolic Ca2+ level (Ca2+ sensitization due to the inhibition of myosin light chain phosphatase). Since GTPγS, an activator of G-protein, inhibits myosin light chain phosphatase activity, G-proteins may be involved in the mechanism of Ca2+ sensitization. Indeed, the Ras-family small G-protein RhoA is activated in smooth muscle after the receptor activation, and plays a crucial role in the mechanism of Ca2+ sensitization.1
The response of various cells to classical growth factors and other mitogens is quite complex, and several possible pathways are activated depending on the stimulus. In most of these pathways, the signal is transmitted via the sequential activation of cytoplasmic protein kinases, leading to the activation of nuclear transcription factors. Mitogen-activated protein kinases (MAPKs) and their substrates have been recognized as central regulators of mitogenic signaling pathways, even in smooth muscle. Activation of the mitogen-activated protein kinase pathway has been suggested to contribute to airway inflammation and airway hyperresponsiveness. The best-characterized mammalian mitogen-activated protein kinases are (1) the 42/44-kDa extracellular signal-regulated kinase (p42/44-ERK); (2) the c-Jun N-terminal kinase (JNK) or stress-activated protein kinase (SAPK); and (3) p38 MAPK. These kinases have also been identified as potential sites of cross-talk between contractile and mitogenic pathways because their activation has been correlated with the mitogenic effects of contractile agonists acting on G protein-coupled receptors. Previous studies have suggested that tyrosine (Tyr) kinases might also play a role in contractile responses evoked by agonists acting on G protein-coupled receptors. Studies of vascular smooth muscle have indicated that mitogen-activated protein kinases may play a role in its contraction. However, few reports have addressed the mechanisms that underlie this process. The aim of the current study was to investigate whether Tyr kinases and mitogen-activated protein kinases are involved in the contractile response of bronchial smooth muscle induced by G protein-coupled receptor stimulation.

Materials and Methods

Animals
Male Wistar rats (6 weeks of age, specific pathogen-free, 180–240 g, Charles River Japan Inc, Kanagawa, Japan) were used. All experiments were performed according to the Guiding Principles for the Care and Use of Laboratory Animals as approved by the Animal Care Committee of Hoshi University (Tokyo, Japan).

Functional Studies of Bronchial Smooth Muscle

The airway tissues from the larynx to the lungs were removed under chloral hydrate (400 mg/kg, intraperitoneal administration) anesthesia. Two mm-wide sections of the left main bronchus were isolated, and the resultant tissue ring preparations were suspended in a 5-ml organ bath at a resting tension of 1.0 g. The isometric contraction of the circular smooth muscle was measured with a force-displacement transducer (TB-612T, Nihon Kohden, Tokyo, Japan). The organ bath contained modified Krebs-Henseleit solution with the following composition (mM): NaCl 118.0, KCl 4.7, CaCl₂ 2.5, MgCl₂ 1.2, NaHCO₃ 25.0, KH₂PO₄ 1.2 and glucose 10.0 (pH 7.4). The buffer solution was maintained at 37°C and oxygenated with 95% O₂-5% CO₂. During an equilibration period in the organ bath, the tissues were washed four times at 15-min intervals and equilibrated slowly to a baseline tension of 9.8 mN. After the equilibration period, a concentration-response curve to carbachol (CCh; 3 nM-100 μM) was constructed cumulatively. Each inhibitor (SP600125, genistein, ST638, U0126 and SB202190) was added 30 min before the first addition (3 nM) of CCh, and was present throughout the experiment. Inhibitor concentrations were chosen on the basis of concentrations previously shown to be effective in similar preparations. A concentration-response curve to orthovanadate sodium (orthovanadate; 3 μM-10 mM) was also constructed cumulatively. Each inhibitor (SP600125, genistein, ST638, U0126 and SB202190) was again added 30 min before the first addition (3 μM) of cumulatively added orthovanadate, and was present throughout the experiment. The levels of tension obtained with normal Krebs-Henseleit (basal tension) and the maximal contraction induced by CCh (Emax; 8.9 ± 0.65 nN) were assigned to be 0% and 100%, respectively. The non-linear interpolation was represented by a sigmoidal curve analysis algorithm based on the variable Hill coefficient from concentration-activity analysis using GraphPad Prism software (GraphPad Software ver. 5.0c, La Jolla, CA).

Protein Extraction
Homogenates of bronchial tissue were prepared by a method described previously with minor modifications. In brief, the airway tissues below the main bronchi were removed and immediately soaked in ice-cold, oxygenated Krebs-Henseleit solution. They were carefully cleaned of adhering connective tissues, blood vessels and lung parenchyma under stereomicroscopy. The bronchial tissue was then equilibrated in oxygenated Krebs-Henseleit solution (37°C) for 60 min with 15-min washout intervals. After equilibration, the tissue segments were stimulated by a concentration-response curve to carbachol (CCh; 1 μM) for 1, 15 or 30 min. SP600125 (30 μM) was added 30 min prior to the application of CCh. The reaction was stopped by rapid freezing in liquid nitrogen, and the tissue was then homogenized in ice-cold T-PER™ Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Rockford, IL). The tissue homogenate was centrifuged (1,000g, 4°C for 1 min), and supernatants were stored at −85°C until use.

Immunoblotting
To quantify the phosphorylated JNK, Western blotting was performed. In brief, the samples (10 μg of total protein per lane) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were then electrophoretically transferred to a polyvinylidene difluoride membrane. Transferred polyvinylidene difluoride membranes were incubated in primary antibodies, either rabbit anti-JNK (1:1000 dilution; Cell Signaling Technology, Beverly, MA) or anti-phosphorylated JNK (p-JNK; 1:1000 dilution; Cell Signaling Technology). The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Ig) G (1:3000 dilution; GE Healthcare, Buckinghamshire, United Kingdom); detection was performed with an enhanced chemiluminescence system. To normalize the p-JNK content to JNK, the ratio of corresponding p-JNK/JNK was calculated as an index of p-JNK.
Statistical Analysis
Statistical analyses were performed using one-way ANOVA. In the case of concentration-contraction curve analysis, means were compared among conditions and over time using two-way repeated measure ANOVA, where both dose and time are repeated factors. Pairwise mean comparisons were judged significant using the Bonferroni/Dunn’s post hoc-test (two-tailed test) using GraphPad Prism software. A value of $P < 0.05$ was considered significant. During the review process several new samples were added to the experiment, but no attempts were made to adjust for the multiple analyses of the data.

Results
Involvement of JNK in CCh-induced Bronchial Smooth Muscle Contraction
To examine the role of mitogen-activated protein kinase(s) in bronchial smooth muscle contraction, the effects of MAPK inhibitors were investigated. The contraction induced by carbachol (CCh) was significantly inhibited by pretreatment with SP600125 ($P < 0.001$), which has been classified as a JNK inhibitor with almost equal inhibitory effects against JNK-1, 2 and 3, but not by pretreatment with the inhibitor of p42/44 ERK activation U-0126 or the p38 MAPK inhibitor SB202190 (fig. 1, A–C). The sustained contraction induced by 1 μM CCh was also inhibited by the application of SP600125 in a concentration-dependent manner (fig. 1, D and E). To examine whether CCh induces the activation of JNK in rat bronchial smooth muscle, immunoblotting was performed using specific antibodies against phosphorylated JNK. The application of CCh produced a significant increase in phosphorylated JNK-1 and -2/3 (fig. 2, A and B; $P < 0.05$). The increases in both phosphorylated JNK-1 and -2/3 induced by CCh were abolished by SP600125 (fig. 2, C and D, $P < 0.05$).

![Fig. 1. Effects of MAPK inhibitors (U0126, SB202190 and SP600125) on the carbachol (CCh)-induced contraction of bronchial rings isolated from rats. 10 μM U0126 (A), 10 μM SB202190 (B), 10 and 30 μM SP600125 (C) were added 30 min before the first addition of CCh. Values are the means ± SEM from six independent experiments, respectively. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. Control (DMSO). Typical traces of the effects of post-treatment with vehicle (DMSO, 0.01–0.3%, D) and SP600125 (1–30 μM, E) on 1 μM CCh-induced contraction in rat bronchial rings.](anesthesiology.pubs.asahq.org)
Involvement of Tyr Kinases in CCh-induced Bronchial Smooth Muscle Contraction

To examine the involvement of Tyr kinases in CCh-induced contraction, the effects of Tyr kinase inhibitors (genistein and ST638) were investigated. The contraction induced by CCh was significantly inhibited in the presence of genistein ($P < 0.001$). Similarly, ST638 also inhibited CCh-induced bronchial smooth muscle contraction in rats ($P < 0.01$) (fig. 3). The contraction induced by CCh was not affected by the pretreatment of an L-type Ca$^{2+}$ channel blocker, nicardipine ($10^{-6}$ M). The phosphorylations of JNKs were not also affected by nicardipine.

Involvement of JNK in Orthovanadate-induced Bronchial Smooth Muscle Contraction

Next, the effects of orthovanadate, a potent Tyr phosphatase inhibitor, on bronchial smooth muscle tension were examined. As shown in figure 4, orthovananate caused development in
Role of the JNK Pathway in Bronchial Contraction

Fig. 3. Effects of tyrosine (Tyr) kinase inhibitors (genistein and ST638) and L-type Ca\(^{2+}\) channel blocker (nicardipine) on the carbachol (CCh)-induced contraction of bronchial rings isolated from rats. Genistein (30 and 100 \(\mu\)M, A) and ST638 (10 and 30 \(\mu\)M, B) were added 30 min before the first addition of 1 \(\mu\)M CCh. Values are the means ± SEM from six (vehicle control, dimethyl sulfoxide; DMSO) and six (Tyr kinase inhibitors) independent experiments, respectively. *\(P<0.05\), **\(P<0.01\) and ***\(P<0.001\) vs. Control (DMSO). Nicardipine (1 \(\mu\)M; C, D) was added 5 min before the addition of CCh. Values are the means ± SEM from five independent experiments, respectively. Typical photographs of effect of nicardipine on the CCh-induced phosphorylated JNK-1 and JNK-2/3 (D).

Involvement of JNK in High K\(^+\) Depolarization-induced Bronchial Contraction

To examine the involvement of Tyr kinase and JNK in the contraction induced by Ca\(^{2+}\) itself, bronchial smooth muscle tissues were stimulated by high K\(^+\) depolarization. The contraction induced by high K\(^+\), which changes the K\(^+\) equilibrium potential to activate L-type Ca\(^{2+}\) channels, is regulated solely by augmentation of the cytosolic Ca\(^{2+}\) concentration.\(^1\) Indeed, the contraction induced by high K\(^+\) was abolished by treatment with nicardipine in rat bronchial smooth muscle (fig. 5A). The contraction induced by high K\(^+\) was also significantly inhibited by genistein (\(P<0.001\); fig. 5B), ST638 (\(P<0.05\); fig. 5C) and SP600125 (\(P<0.001\); fig. 5D). On the other hand, neither SB202190 nor U-0126 affected high K\(^+\)-induced contraction (fig. 5, E and F).

Discussion

We demonstrated here for the first time that JNK activation via Tyr kinase is involved in CCh-induced bronchial smooth muscle contraction in the rat. The contractions induced by CCh and high K\(^+\) were inhibited by a JNK inhibitor, but not...
by a p38 MAPK or p42/44-ERK inhibitor. In rat vascular smooth muscle, the JNK pathway has been shown to contribute to noradrenaline-induced contraction. Noradrenaline also increases the activities of p42/44-ERK and p38 MAPK and induces contraction in vascular smooth muscle, which is inhibited by these kinase inhibitors. 20,21 It has also been reported in vascular smooth muscle that high K⁺-induced contraction was not inhibited by treatment with JNK inhibitors. 22 Thus, the mechanisms for inducing contraction in rat bronchial smooth muscle may be different than those in aortic smooth muscle.

Consistent with the results of the contraction study, JNKs were phosphorylated by the application of CCh in rat bronchial smooth muscle, and this effect was abolished by pretreatment with the JNK inhibitor SP600125. Furthermore, CCh-induced contraction was also inhibited by Tyr kinase inhibitors in this tissue. The contraction induced by the Tyr phosphatase inhibitor, orthovanadate, was inhibited by either genistein or SP600125. Taken together, these findings suggest that the activation of Tyr kinase is an upstream pathway of JNK activation induced by CCh in rat bronchial smooth muscle.

The smooth muscle contraction induced by contractile agonists primarily depends on the phosphorylation of myosin light chain (MLC). Recent studies have revealed that smooth muscle contraction is regulated by cytosolic Ca²⁺-dependent and -independent pathways. The former activates calmodulin (CaM)/myosin light chain kinase (MLCK) to phosphorylate MLC, while the latter inhibits MLC phosphatase (MLCP) to augment MLC phosphorylation. 1 In the present study, high K⁺ (10–60 mM)-induced contraction of rat bronchial smooth muscle was abolished by nicardipine, an L-type Ca²⁺ channel blocker. The contraction induced by high K⁺ was also inhibited by both Tyr kinase and JNK inhibitors. However, the contraction induced by CCh was not inhibited by nicardipine. These findings are consistent with a previous report stating that acetylcholine-induced bronchial smooth muscle contraction was not caused by L-type Ca²⁺ channel activation. 23 This result shows that the inhibition of Tyr kinase/JNK hinders the intracellular Ca²⁺-induced pathway, but not the L-type Ca²⁺ channel itself. These findings suggest that the activation of Tyr kinase/JNK may depend on intracellular Ca²⁺, which directly or indirectly leads to activation of the Ca²⁺/CaM/MLCK pathway. One possible mechanism of Tyr kinase/JNK-mediated Ca²⁺ dependent signaling may be related to Ca²⁺ dependent phosphoinositide 3-kinase class IIα isoform-mediated signaling pathway. 24 Further examination is necessary to clarify the point.

In the past, especially in vascular smooth muscles, it has been reported that tyrosine kinase inhibitors reduced both the constriction and increase in intracellular Ca²⁺ caused by agents such as norepinephrine, phenylephrine, endothelin-1, and angiotensin II. 25–28 In contrast, it has also reported
that tyrosine kinase inhibitors induced the vasodilator effect without preventing intracellular Ca²⁺ mobilization. Thus, effects of tyrosine kinase inhibitors on intracellular Ca²⁺ mobilization may be variable in each animal, organ, chemical and experimental condition, indicating that effects of genistein and ST638 on intracellular Ca²⁺ movement should be investigated in bronchial smooth muscles, although we did not examine them in the current study. We have to confirm the effect of tyrosine kinase inhibitors on Ca²⁺ mobilization in stimulant-induced bronchial smooth muscle contractions.

We also examined the roles of Tyr kinase/JNK in the MLCP inhibition pathway (Ca²⁺-independent pathway).

Sodium fluoride (NaF), an activator of G-proteins, is known to activate the trimeric G protein and RhoA pathways, resulting in the inhibition of MLCP activity and an increase in MLC phosphorylation. It has been reported that a RhoA inhibitor, C3 toxin, inhibits ACh-induced contraction in rat bronchial smooth muscle. Our previous study demonstrated that NaF-induced contraction was markedly inhibited by Y-27632, a Rho kinase inhibitor. In the present study, the JNK inhibitor, SP600125, significantly inhibited NaF-induced contraction. These results suggest that the Tyr kinase/JNK pathway may be associated not only with the MLCK pathway but also with the MLCP

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**Fig. 5.** Effects of Tyr kinase and MAPK inhibitors (genistein, ST638, SP600125, SB202190 and U0126) on the high K⁺ depolarization-induced contraction of bronchial rings isolated from rats. Typical trace of the effect of nicardipine on high K⁺-induced contraction in bronchial smooth muscle (A). Genistein (30 and 100 μM, B), ST638 (3 and 10 μM, C), SP600125 (10 and 30 μM, D), SB202190 (10 μM, E), and U0126 (10 μM, F) were added 30 min before the first addition of K⁺. Values are the means ± SEM from four independent experiments, respectively. *P < 0.05 and ***P < 0.001 vs. Control (DMSO).
inhibition pathway. Therefore, the Tyr kinase/JNK pathway may be involved in both Ca\(^{2+}\)-dependent and -independent pathways. On the other hand, the contraction induced by calyculin-A, a potent MLCP inhibitor, was not affected by SP600125. Thus, it seems unlikely that the Tyr/JNK pathway affects the smooth muscle contractile apparatus, such as the activity of actomyosin ATPase.

In conclusion, the Tyr kinase/JNK pathway is responsible for CCh-induced bronchial smooth muscle contraction. Strategies to inhibit the Tyr kinase/JNK pathway may represent a novel therapeutic approach for diseases involving airway obstruction, such as asthma and chronic obstructive pulmonary disease.

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Role of the JNK Pathway in Bronchial Contraction

Fig. 7. Carbachol (CCh, 1 μM for 15 min), sodium fluoride (NaF, 10 mM for 15 min), high K+ (60 mM for 3 min), and othovanadate-induced (1 mM for 20 min) phosphorylation of JNKs in rat bronchial smooth muscle. Phosphorylation of JNK-1 and JNK-2/3 was revealed by Western blotting. Typical photographs of CCh-induced phosphorylated-JNK/total JNK) represents the fold increase from Naive (B, C). Values are means ± SEM from four independent experiments. *P < 0.05, **P < 0.01 vs. Naive.

References


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The McMechans’ 1937 Loving Cup

Among the many treasures that the Wood Library-Museum secures for the International Anesthesia Research Society, certainly one of the greatest is the loving cup (left) that the IARS had dedicated in 1937 to its founders (right), Dr. Francis “Frank” McMechan and his wife Laurette. Seventeen years after her husband had succumbed to complications of crippling rheumatoid arthritis, in 1956, the Widow McMechan retired from their Ohio home to southern California. The loving cup disappeared for decades until it was curatorially recovered from New Mexico in 2011. Dusting off the long-lost trophy revealed the part of the inscription thanking the McMechans for their “devoted services and splendid achievements for the organization, economics, research, practice, teaching, journalism and fellowship of the specialty of anesthesia....” (Copyright © the American Society of Anesthesiologists, Inc.)

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