

BASIC MECHANISMS

Store-Operated Ca^{2+} Entry is Involved in Transforming Growth Factor- β 1 Facilitated Proliferation of Rat Airway Smooth Muscle Cells

YA-DONG GAO*, JUN-WEN ZHENG, PING LI, MING CHENG, AND JIONG YANG

Department of Respiratory Medicine, Zhongnan Hospital, Wuhan University, Wuhan, China.

Objective. To investigate the role and underlying mechanisms of store-operated Ca^{2+} entry (SOCE) in mediating the promoting effect of transforming growth factor (TGF)- β 1 on the proliferation of airway smooth muscle cells (ASMCs). **Methods.** Rat bronchial smooth muscle cells were cultured as we described previously. The intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) of ASMCs was measured by laser confocal microscope Ca^{2+} fluorescence imaging with Fluo-3/AM. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and p27 expression assay were used to determine the proliferation rate of ASMCs. **Results.** We demonstrated that TGF- β 1 (10 ng/ml) increased basal (Ca^{2+})_i level, $[\text{Ca}^{2+}]_i$ rise induced by thapsigargin-induced Ca^{2+} release and SOCE in rat ASMCs. This effect of TGF- β 1 on SOCE was not inhibited by glucocorticoid dexamethasone (DXM, 100 nM), antioxidant α -tocopherol (100 μM), and intermediate-conductance Ca^{2+} -activated K^+ channels (IK_{Ca}) inhibitor charybdotoxin (100 nM), suggesting that reactive oxygen species and IK_{Ca} channels might not mediate the effect of TGF- β 1. TGF- β 1 slightly increased the expression of Orai1 and STIM1, two important molecules involved in the molecule component and regulation of SOC channels, in the presence of 10% fetal bovine serum (FBS). The proliferation of ASMC stimulated with 2.5% FBS was promoted by TGF- β 1, and partly inhibited by non-specific Ca^{2+} channel blocker SKF-96365 (10 μM) and Ni^{2+} (100 μM). DXM, α -tocopherol, and charybdotoxin had no effect on the proliferation promoted by TGF- β 1. **Conclusion.** TGF- β 1 promotes ASMC proliferation partly through increasing the expression and activity of SOC channels.

Keywords airway smooth muscle cells, proliferation, store-operated Ca^{2+} entry, transforming growth factor- β 1

INTRODUCTION

Dysfunction of airway smooth muscle plays a central role in the pathogenesis of airway hyperresponsiveness (AHR), which is characterized by excessive narrowing of airway upon exposure to constrictive agonists (1). Increased airway smooth muscle mass has been shown to contribute to airway remodeling, which results in persistent airflow limitation (2). The underlying mechanisms of airway remodeling are not well understood.

Previous studies have shown that transforming growth factor- β 1 (TGF- β 1) is involved in the pathophysiology of asthma. Elevated TGF- β 1 expression was seen in the airway submucosa of asthmatic individuals compared with normal subjects, and the increase of TGF- β 1 was correlated with the severity of the disease (3). It was shown that the majority of TGF- β 1-positive cells in asthmatic tissues were eosinophils (4). Additional sources of TGF- β 1 in the airway were epithelia (5) and airway smooth muscle cells (ASMCs) (6). A role of TGF- β 1 in airway remodeling has been well documented. It was shown that the expression of TGF- β 1 in ASMCs was significantly increased in asthma patients (7). But the effect of TGF- β 1 on the proliferation of ASMCs is still conflicting (7–9). In addition, TGF- β 1 also increases the secretion of matrix metalloproteinase (10) and production of extracellular matrix (11) in ASMCs, suggesting a new mechanism

of TGF- β 1 in airway remodeling. Moreover, TGF- β 1 reduces the expression of β -adrenergic receptors in airway smooth muscle, which then attenuates the therapeutic effects of β 2 agonists. All these data suggest that TGF- β 1 is a multifunctional growth factor involved in the functional abnormality of airway smooth muscle in asthma.

Ca^{2+} signaling plays an important role in the regulation of airway smooth muscle functions, such as contraction, proliferation, and cytokine secretion (12). Store-operated Ca^{2+} entry (SOCE) is the major Ca^{2+} influx pathway of ASMCs. Store-operated Ca^{2+} channels will be activated upon depletion of intracellular sarcoplasmic reticulum Ca^{2+} store. SOCE mediates multiple functions of ASMCs such as contraction and proliferation (12). Our previous study showed that knockdown of STIM1 and Orai1, two important regulatory and component molecules of SOC channels, decreased SOC activity, and proliferation of rat ASMCs (13). It was shown that TGF- β 1 potentiates Ca^{2+} release induced by bradykinin in airway smooth muscle by increasing the surface expression of bradykinin B2 receptor (14). In addition, TGF- β 1 increases the expression Ca^{2+} -activated K^+ channels $\text{K}_{\text{Ca}3.1}$ in human ASMCs (15), which then facilitates Ca^{2+} influx by providing driving force. Effect of TGF- β 1 on Ca^{2+} signaling was also demonstrated in other cell types, e.g. facilitating Ca^{2+} influx in human lung fibroblasts (16), regulating the expression of IP3 receptors in vascular smooth muscle cells (17), and ryanodine receptors in cardiomyocytes (18). Whether TGF- β 1 has a

*Corresponding author: Ya-Dong Gao, Department of Respiratory Medicine, Zhongnan Hospital, Wuhan University, Donghu Road 169, 430071 Wuhan, China; Tel: +86 27 6781 3327; Fax: +86 27 6875 8766; E-mail: gaoyadong@gmail.com

regulatory effect on SOCE is not reported so far. In the present study, we investigated the effect of TGF- β 1 on SOCE in cultured rat ASMCs and the possible underlying mechanisms.

METHODS

Materials and Solutions

Recombinant human TGF- β 1 was obtained from Peprotech (Rocky Hill, USA). Fluo-3/AM and pluronic F-127 were from Dojindo laboratories (Kumamoto, Japan). Other chemicals and charybdotoxin (CTX) were obtained from Sigma-Aldrich (St. Louis, MO, USA). TGF- β 1, CTX, and LaCl_3 were dissolved in pure water and other chemicals in dimethyl sulfoxide (DMSO) as stock solutions. The final concentration of DMSO was less than 0.2% at which it had no influence on Ca^{2+} signal.

Preparation of ASMCs

This study was approved by Animal Care Committee of Wuhan University. ASMCs were isolated from male Sprague-Dawley rats as we described previously (19). Briefly, rat bronchi were isolated under sterile conditions. After removing the connective tissue and epithelia carefully, the smooth muscle strips were cut into small pieces and digested in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA)-containing 1 mg/ml type I collagenase and 2 mg/ml papain (both from Invitrogen, USA) at 37°C for 30 min. The cells were dispersed and centrifuged at 200 G for 10 min; the pellets were collected and resuspended in DMEM. The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 U/ml penicillin and 0.1 mg/ml streptomycin (both from Invitrogen). Cells between 3 to 6 passages were used for experiments. All experiments were repeated in cells from at least 3 different rats.

Intracellular Ca^{2+} Measurement

Cytosolic Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) was determined with laser confocal microscope Ca^{2+} fluorescence measurement as we described previously (19). ASMCs grown in glass coverslips were subjected to different treatments for 24 h and then incubated with 5 μM Fluo-3/AM and 0.1% pluronic F-127 in physiological salt solution (PSS: 140 mM NaCl, 10 mM KCl, 1 mM MgCl_2 , 2 mM CaCl_2 , 10 mM HEPES, 10 mM Glucose, pH 7.2) at 37°C for 30 ~ 45 min. After de-esterification of Fluo-3/AM at room temperature (22°C) for 15 min, coverslips with ASMCs were inserted to the bottom of a recording chamber containing 1 ml Ca^{2+} -free PSS. 10 μM nifedipine was included in the Ca^{2+} -free PSS to block voltage-dependent Ca^{2+} channels (VDCC). Cells were visualized with an inverted microscope (Leica DM IRE2, Wetzlar, Germany) with a 20 $\times$ objective lens. The mean Ca^{2+} fluorescence intensity (FI) in the Region of Interest (ROI) was measured with a Leica confocal system (TCS SP2 AOBS MP, Wetzlar, Germany) with an Ar-Kr laser.

15–20 cells were measured simultaneously at every coverslip. The fluorescence was excited at 488 nm and emitted at 530 nm. ROI was defined along the visible boundary of the cells. All the parameters during the recording were kept constant. Ionomycin (10 μM) was added to the bath solution after reintroduction of 2 mM Ca^{2+} to maximize the Ca^{2+} influx and determine the maximal FI (F_{max}). The $[\text{Ca}^{2+}]_i$ was calculated according to the following formula:

$$[\text{Ca}^{2+}]_i = K_d(F - F_{\text{min}})/(F_{\text{max}} - F)$$

K_d for Fluo-3 at wavelength 530 nm is 430 nM. F is the measured FI, F_{min} represents FI when $[\text{Ca}^{2+}]_i$ is at 0. Changes in $[\text{Ca}^{2+}]_i$ was indicated as $\Delta[\text{Ca}^{2+}]_i$.

MTT Proliferation Assay

After reaching confluence, ASMCs were incubated in serum-free medium for 48 h, and then transferred to 96-well plates with a density of 0.5×10^4 cells/well. Cells were then cultured in 1% or 2.5% FBS-containing DMEM and treated with TGF- β 1 (10 ng/ml) or appropriate test reagents for 24 h. Previous studies showed that the maximal effect of TGF- β 1 on proliferation was observed with the concentration of 10 ng/ml (7), thus we used this concentration during our experiments. The proliferation rates of rat ASMCs were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. After washing the cells for two times with PBS, 20 μl of MTT (5 g/L) was added to each well, and cells were then incubated at 37°C for 4 h. MTT was then removed and 150 μl DMSO was added to each well. After incubation for 10 min, the optical absorbency at wavelength 570 nm was determined by a spectrophotometer (GENios VA 200, TECAN, Austria). In certain range, the reduced MTT is proportional to the cell numbers.

p27 Protein Expression Assay

Whole cell protein extracts were prepared from rat ASMCs in different groups. Cells were washed twice and lysed at 4°C in a lysis buffer containing 1% Triton X-100, 150 mM NaCl, and 20 mM Tris (pH7.5) (Beyotime, China). SDS-PAGE Sample loading buffer (5X, Beyotime, China) was added to the cell lysates at a 1:4 ratio (v/v), and the lysates were then boiled for 5 min. Protein extracts (50 μg) were subjected to electrophoresis on SDS-PAGE gel and subsequently transferred to polyvinylidene fluoride membrane (Millipore). The membranes were blocked with TBS-T buffer containing 5% non-fat dried milk at room temperature for 1 h and probed with monoclonal primary antibody specific for rat p27 (Santa Cruz Biotechnology, mouse 1:200) overnight at 4°C, followed by goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, 1:2000) at room temperature for 1 h. BeyoECL Plus (Beyotime, China) and Kodak X-OMAT BT film were used to visualize the target

protein. Quantification of bands was achieved by optical density using Alpha software.

mRNA Expression Assay

The expression of KCNN4 (GenBank access No.: NM_023021.1) on rat ASMCs was assessed by reverse transcription polymerase chain reaction (PCR). Briefly, total RNA was isolated using TRIZOL Reagent (QIAGEN, Valencia, CA) and the cDNA was synthesized from 1 μ g total RNA using ReverTra Ace RT kit (TOYOBO, Osaka, Japan). The primers for rat KCNN4 were: forward 5'-CTCAACCAAGTCCGATTCCG-3'; reverse 5'-GCATTGACAGCCTGCCTCTC-3'. β -actin was used as endogenous control and the specific primers for it were: forward 5'-CGTTGACATCCGTAAGACCTC-3'; reverse 5'-TAGGAGCCAGGGCAGTAATCT-3'. The cycling parameters were: 94°C for 30 s, 56°C for 30 s and 72°C for 1 min for 35 cycles. The PCR production was subjected to

electrophoresis on 1.5% agarose gel and visualized with GoldenView under UV.

Real-time quantitative PCR was used to determine the expression of Orai1 and STIM1 mRNA in rat ASMCs by using SYBR Premix Ex Taq™ (Takara, Tokyo, Japan) as we described previously (19). Specific primers for rat Orai1 is: forward 5'-CCATAAGACGGACCGACAGT-3', reverse 5'-GGGAAGGTGAGGACTTAGGC-3'; rat STIM1: forward 5'-AGCTCCTGGTATGCTCCTGA-3', reverse 5'-TATTTTCTCAGCCCCCTCCT-3'. β -actin gene was used as an internal control and the primers were: forward 5'-AGCCATGTACGTAGCCATCC-3'; reverse 5'-ACCCTCATAGATGGGCACAG-3'. The cycling parameters were: 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s for 40 cycles. The relative expression of the target genes was calculated using the comparative method (Δ Ct, where the Ct indicates the cycle number threshold at which amplification is exponential). The

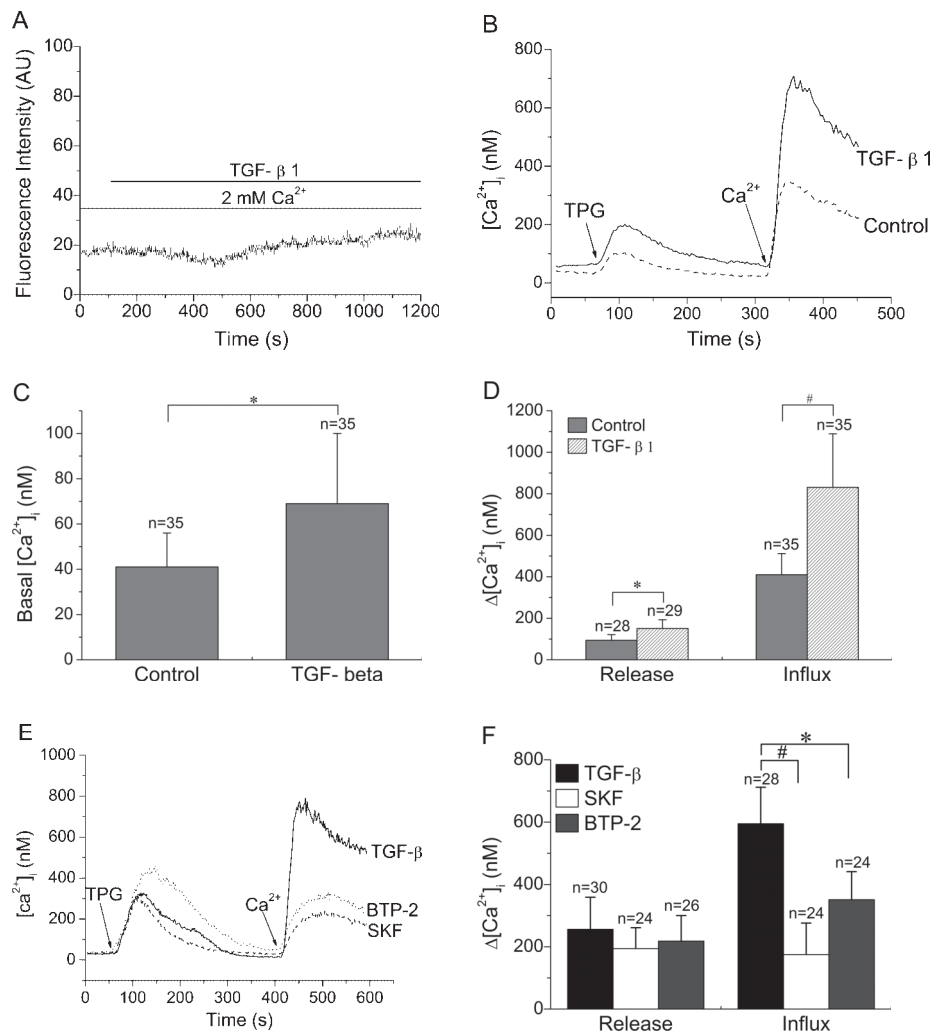


FIGURE 1.—Effect of TGF- β 1 on basal $[Ca^{2+}]_i$, Ca^{2+} release, and influx in ASMCs. ASMCs grown on glass coverslips were cultured in 10% FBS-containing DMEM for 24 h, and then loaded with Fluo-3/AM. Ca^{2+} fluorescence was measured with laser confocal microscope. **A:** acute application of TGF- β 1 (10 ng/ml) did not induce significant Ca^{2+} influx. **B:** treatment of ASMCs with TGF- β 1 (10 ng/ml) for 24 h increased Ca^{2+} signal in ASMCs. **C:** summarized data showing basal $[Ca^{2+}]_i$ in TGF- β 1 24 h-treated and control ASMCs. **D:** summarized data showing Ca^{2+} response induced by thapsigargin (TPG, 1 μ M) and subsequent store-operated Ca^{2+} influx upon reintroduction of Ca^{2+} into bath solution. **E:** Ca^{2+} influx promoted by TGF- β 1 treatment was partly inhibited by SKF-96365 (10 μ M). **F:** summarized data showing the effect of SKF-96365 and SOC channel blocker BTP-2 (100 nM) on TPG-induced Ca^{2+} release and influx in TGF- β 1-treated ASMCs. Data were means $\pm$ SD. *n* on each bar represent numbers of tested cells. **p* < .05, #*p* < .01.

primer efficiencies were assessed by plotting a cDNA standard curve initially to validate to use this method of analysis. The primer efficiencies of each assay were all approximately equal.

Statistical Analysis

All the experiments were repeated on ASMCs from at least 3 different rats. *n* represents cell numbers that were tested in each experiments. Data were means $\pm$ SD or means $\pm$ SE, as indicated in the figure legends. Results were analyzed by Student's *t*-test or one-way ANOVA. Differences were significant when *p* < .05.

RESULTS

TGF- β 1 Promoted Ca^{2+} Release and Store-Operated Ca^{2+} Influx

We firstly determined the acute effect of TGF- β 1 on constitutive Ca^{2+} influx in ASMCs. In 2 mM Ca^{2+} -containing bath solution, application of TGF- β 1 (10 ng/ml) for about 20 min did not elicit obvious Ca^{2+} influx, as shown in Figure 1A, suggesting that TGF- β 1 had no acute effect on Ca^{2+} signal of ASMCs. We then investigated the effect of TGF- β 1 on Ca^{2+} signal by extending the incubation time to 24 h. TGF- β 1 treatment significantly increased the basal $[\text{Ca}^{2+}]_i$ of ASMCs (Figure 1B). Under control condition, the basal $[\text{Ca}^{2+}]_i$ was 40 ± 14 nM. Treatment of TGF- β 1 for 24 h increased basal $[\text{Ca}^{2+}]_i$ about 50% (to 69 ± 41 nM) (Figure 1C). The sarcoplasmic/endoplasmic reticulum

Ca^{2+} -ATPase (SERCA) inhibitor thapsigargin (TPG) was used to deplete Ca^{2+} store by passively releasing Ca^{2+} from the endoplasmic reticulum / sarcoplasmic reticulum (ER/SR). As shown in Figure 1B and D, in Ca^{2+} -free bath solution, the mean Ca^{2+} release response to TPG (1 μM) was 151 ± 42 nM in TGF- β 1-treated ASMCs, which is higher than that of control cells (94 ± 27 nM). After TPG-induced Ca^{2+} transient had been returned to base level, reintroduction of Ca^{2+} into bath solution elicited another large increase of $[\text{Ca}^{2+}]_i$, representing SOCE (Figure 1B). TGF- β 1 treatment increased SOCE about one-fold (831 ± 258 nM vs. control of 410 ± 102 nM) (Figure 1B and D). In addition, TGF- β 1-promoted SOCE was inhibited by non-specific Ca^{2+} channel blocker SKF-96365 (10 μM) and SOC channel relatively selective inhibitor BTP-2 (100 nM) (Figure 1E and F). These results suggested that long-term exposure to TGF- β 1 increased Ca^{2+} signal of ASMCs.

Effects of Glucocorticoids on TGF- β 1-Promoted SOCE

Glucocorticoids are currently the mainstay of asthma therapy. We then observed the effect of dexamethasone (DXM) on Ca^{2+} signals promoted by TGF- β 1. ASMCs were incubated with TGF- β 1 (10 ng/ml) in the presence or absence of DXM (100 nM). After 24 h, Fluo-3/AM fluorescence Ca^{2+} measurement showed that the basal $[\text{Ca}^{2+}]_i$, TPG-induced Ca^{2+} release responses, and SOCE were not different between two groups (Figure 2A–C). Thus, glucocorticoids were not effective in the regulation of Ca^{2+} signal that was altered by TGF- β 1.

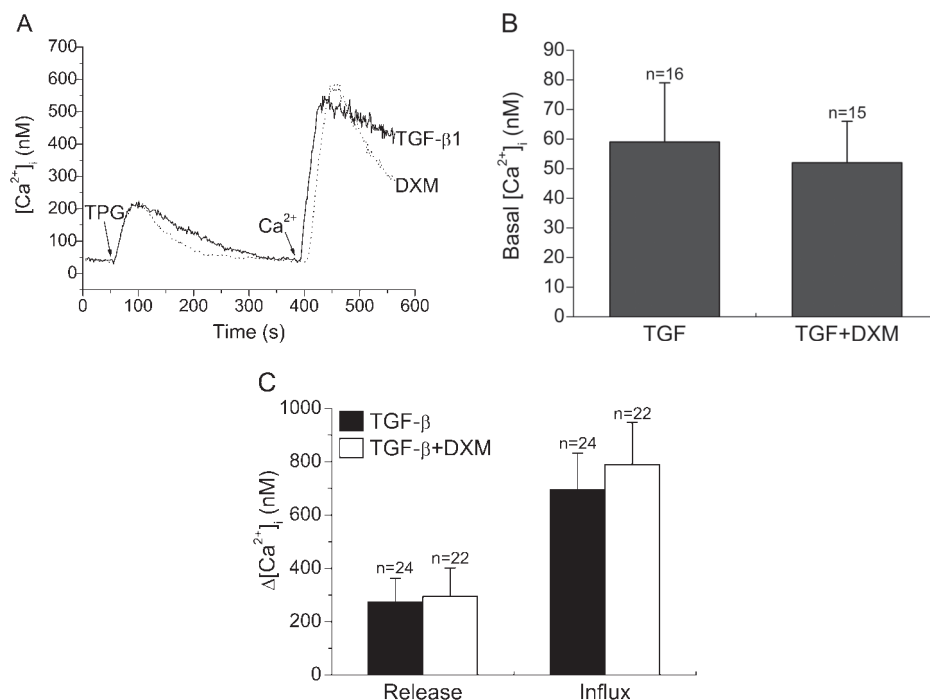


FIGURE 2.—Effect of dexamethasone on Ca^{2+} signal in TGF- β 1-treated ASMCs. **A**: time-course recording showing TPG (1 μM) —induced Ca^{2+} responses in cells that were treated with TGF- β 1 (10 ng/ml) alone or in the presence of dexamethasone (DXM, 100 nM) for 24 h. **B**: summarized data showing basal $[\text{Ca}^{2+}]_i$ in TGF- β 1— and TGF- β 1/DXM—treated ASMCs. **C**: summarized data showing TPG-induced Ca^{2+} release and store-operated Ca^{2+} influx in ASMCs treated with TGF- β 1 or TGF- β 1/DXM. Data are means $\pm$ SD. *n* on each bar represent numbers of tested cells.

Role of IK_{Ca} Channel in TGF- β 1-Promoted SOCE

Previous studies showed that TGF- β 1 promoted the expression of intermediate-conductance Ca^{2+} -activated K^+ channel ($K_{Ca3.1}$, KCNN4) in human ASMCs (15). Since activation of IK_{Ca} will hyperpolarize cell membrane and provide driving force for Ca^{2+} influx, we investigated whether IK_{Ca} mediates the effect of TGF- β 1 on SOCE. KCNN4 mRNA was detected in rat ASMCs, as shown in Figure 3A. Ca^{2+} fluorescence imaging showed that in TGF- β 1-treated ASMCs, IK_{Ca} inhibitor CTX (100 nM) did not affect TPG-induced Ca^{2+} release (not

shown). In TGF- β 1-treated cells, CTX did not change the peak value of store-operated Ca^{2+} influx induced by TPG (1 μ M) (Figure 3B). Moreover, CTX also did not accelerate the Ca^{2+} plateau decay rate in TGF- β 1-treated ASMCs (Figure 3B and C). In TGF- β 1-treated cells, $[Ca^{2+}]_i$ decayed to $(70 \pm 11)\%$ and $(66 \pm 11)\%$ of peak level within 4 min in the absence and presence of CTX, respectively (Figure 3B and C). Similar results were obtained in control cells (Figure 3C). These data suggested that IK_{Ca} was not involved in the modulation of SOCE by TGF- β 1 in rat ASMCs.

Effect of Antioxidant α -Tocopherol on TGF- β 1-Promoted Ca^{2+} Influx

TGF- β 1 was shown to increase the production of reactive oxygen species (ROS) in ASMCs through up-regulation of nicotinamide adenine dinucleotide phosphate (NADPH) and inhibition of the expression of antioxidant enzymes, thus leads to oxidant/antioxidant imbalance (20). Hydrogen peroxide (H_2O_2) is the main source of ROS (16). Recent studies demonstrated that H_2O_2 induces Ca^{2+} mobilization from IP_3 receptor- and ryanodine receptor-sensitive Ca^{2+} store (21, 22), which then activates store-operated Ca^{2+} channels (21). Therefore, TGF- β 1 may promote SOCE indirectly through the generation of H_2O_2 . α -tocopherol (Vitamin E) is a common antioxidant that was shown to be effective in alleviating critical features of asthma in a mouse model (23). We then investigated the effect of α -tocopherol on SOCE of ASMCs. α -tocopherol (100 μ M) treatment for 24 h had no effect on basal $[Ca^{2+}]_i$ in both control and TGF- β 1-treated ASMCs (Figure 4A and C). Interestingly, α -tocopherol significantly decreased Ca^{2+} transient induced by TPG in both group of ASMCs (Figure 4 A–D). Nevertheless, the mean peak amplitude of Ca^{2+} influx was not significantly changed after treatment with α -tocopherol (Figure 4A–D). These data suggested that oxidative stress was not involved in TGF- β 1-promoted SOCE.

Effect of TGF- β 1 on the Expression of *STIM1* and *Orai1*

We then determined whether TGF- β 1 promotes Ca^{2+} influx by increasing the expression of *STIM1* and *Orai1*, two essential components of store-operated Ca^{2+} channels. Our real-time PCR experiments showed that in FBS-free medium, TGF- β 1 (1 and 10 ng/ml) had no effect on the expression of *STIM1* and *Orai1* at mRNA level (Figure 5A). In 10% FBS-containing DMEM, low concentration of TGF- β 1 (1 ng/ml) had no effect on the expression of both genes. TGF- β 1 (10 ng/ml) treatment significantly increased the mRNA expression of *Orai1* and *STIM1* in comparison to control cells ($p < .05$) (Figure 5B).

Role of SOCE in TGF- β 1-Stimulated Proliferation of ASMCs

To investigate whether SOCE is involved in the proliferation of ASMCs promoted by TGF- β 1, we examined serum-stimulated proliferation of rat ASMCs with MTT assay and p27 expression assay. As shown in Figure 6A and D, TGF- β 1 (10 ng/ml) had no significant effect on the

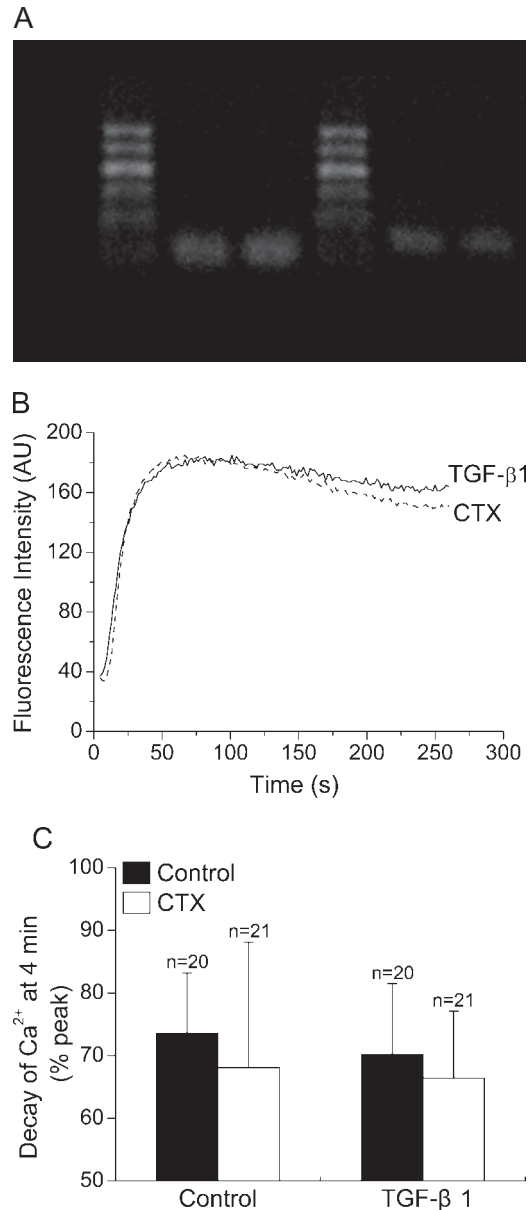


FIGURE 3.—Possible role of IK_{Ca} channels in mediating the effect of TGF- β 1 on SOCE. **A:** KCNN4 expression in rat ASMCs (PCR), C, control cells; T, TGF- β 1-treated cells. **B:** effect of IK_{Ca} channel blocker charybdotoxin (CTX, 100 nM) on Ca^{2+} fluorescence decay rate of TPG-induced Ca^{2+} influx in TGF- β 1-treated ASMCs. **C:** Percentages of Ca^{2+} fluorescence decay at 4 min after Ca^{2+} influx reached to the peak in control and TGF- β 1-treated ASMCs. Ca^{2+} influx was induced by addition of 2 mM Ca^{2+} into bath solution after TPG-induced Ca^{2+} release has been returned to baseline (not shown). Data are means $\pm$ SD. n on each bar represent numbers of tested cells.

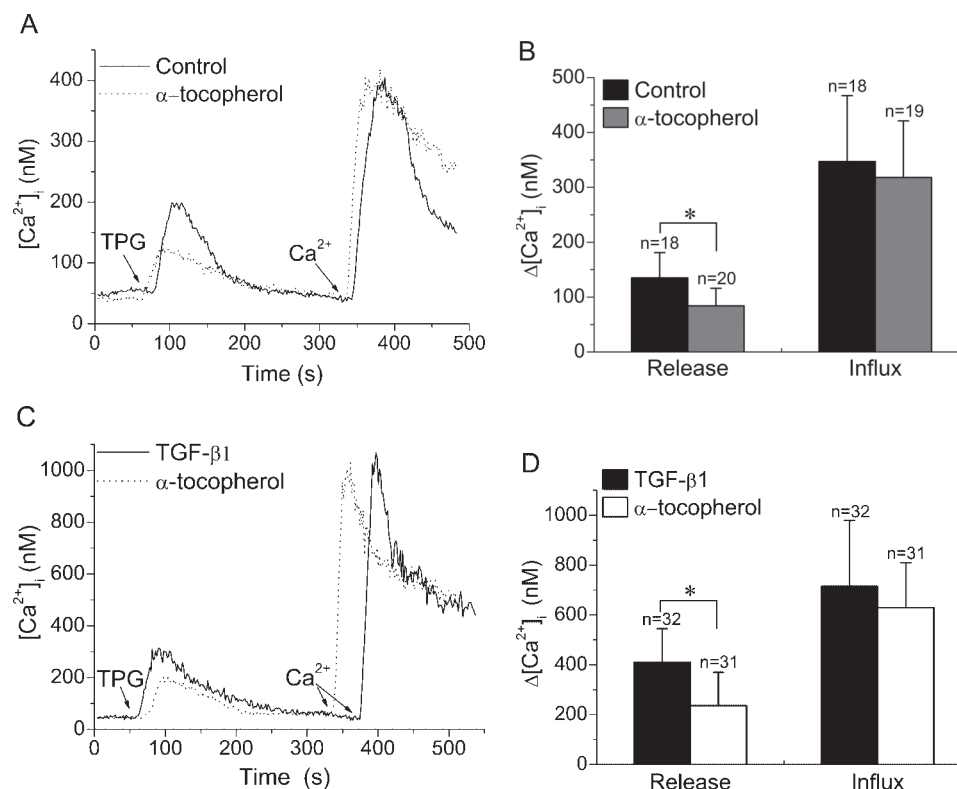


FIGURE 4.—Effect of α -tocopherol on Ca^{2+} release and store-operated Ca^{2+} entry induced by TPG. **A:** In control ASMCs, α -tocopherol (100 μM) treatment for 24 h decreased TPG-induced Ca^{2+} transient but not store-operated Ca^{2+} entry upon reintroduction of Ca^{2+} into bath solution. TPG (1 μM) and Ca^{2+} were added into bath solution at arrow-indicated time. **B:** Summarized data showing the effect of α -tocopherol treatment on Ca^{2+} release and store-operated Ca^{2+} entry induced by TPG in control cells. **C:** In TGF- β 1-treated ASMCs, α -tocopherol treatment for 24 h reduced TPG-induced Ca^{2+} transient, but not store-operated Ca^{2+} entry. **D:** Summarized data showing the effect of α -tocopherol treatment on TPG-induced Ca^{2+} release and SOCE in TGF- β 1-treated cells. Data are means $\pm$ SD. * $p < .01$. n on each bar represent numbers of tested cells.

proliferation of ASMCs stimulated with 1% FBS, even though a trend of increased MTT reduction rate and decreased p27 expression was observed in TGF- β 1-treated cells. In the presence of 2.5% FBS, the growth of ASMCs was significantly promoted by TGF- β 1 ($p < .01$). This promoting effect of TGF- β 1 was inhibited by non-specific Ca^{2+} channel blocker Ni^{2+} (100 μM) and SKF-96365 (10 μM), but not by La^{3+} (100 μM) (Figure 6B and E). We also checked the effect of the relatively selective SOC blocker BTP-2 on the proliferation of ASMCs in 2.5% FBS. As shown in Figure 6C and F, in 2.5% FBS-containing medium, BTP-2 (100 nM) decreased the MTT reduction rate and increased p27 expression in ASMCs stimulated with TGF- β 1. Taken together, these results confirmed that SOCE was involved in TGF- β 1 promoted proliferation of ASMCs.

We then observed the effects of IK_{Ca} channel blocker CTX (100 nM), antioxidant α -tocopherol (100 μM) and glucocorticoid DXM (100 nM) on the proliferation of ASMCs under stimulation with 2.5% FBS in control and TGF- β 1-treated cells. As shown in Figure 7A and B, DXM had no effect on the proliferation of ASMCs under serum stimulation. Both α -tocopherol and CTX slightly increased the proliferation of ASMCs stimulated with 2.5% FBS, although without statistical significance. In the presence of TGF- β 1, there were no differences of MTT reduction rate and p27 expression among these different treated cells.

DISCUSSION

TGF- β 1 is thought to play important roles in regulating airway inflammation and remodeling of asthma (10, 24, 25). In the present study, we present evidences to show that TGF- β 1 modulates Ca^{2+} homeostasis of rat ASMCs by promoting SOCE, and this alteration in Ca^{2+} signaling contributes to its effect on ASMC proliferation.

Different mechanisms have been suggested to be involved in the regulation of Ca^{2+} homeostasis by TGF- β 1, but its effect on SOC channels was not illustrated. In this study, we used SERCA inhibitor thapsigargin to passively deplete Ca^{2+} store, in that TPG-induced Ca^{2+} release is less influenced by the activity of IP3R, ryanodine receptor, or phospholipid C (PLC), which might be changed after treatment of ASMCs with TGF- β 1. We found that the TPG-induced Ca^{2+} transient was larger in TGF- β 1-treated ASMCs, similar to what we have observed in IL-13-treated ASMCs (19). Interestingly, previous studies showed that TGF- β 1 increased the expression of sarcoplasmic reticulum in cardiomyocytes (18, 26). Thus, TGF- β 1 may increase Ca^{2+} release by modulating the Ca^{2+} store content or SERCA activity of ASMCs.

We demonstrated here that Ca^{2+} entry through SOC channels was significantly increased by TGF- β 1. This effect should be partly ascribed to increased Ca^{2+} release

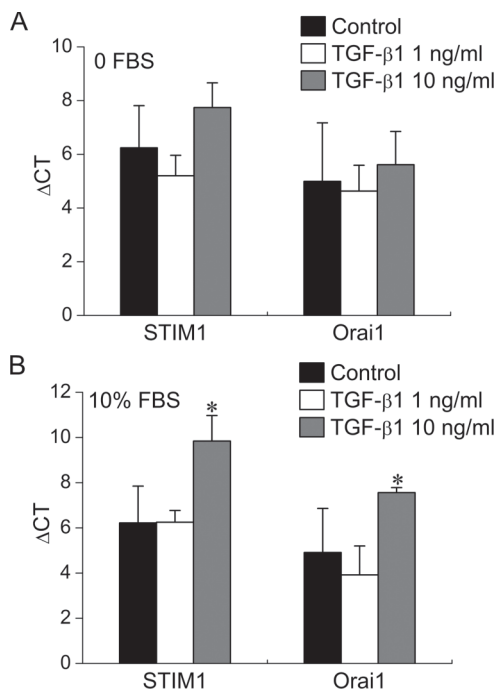


FIGURE 5.—Effect of TGF- β 1 on the expression of STIM1 and Orai1. Rat ASMCs grown in 6-well plates were synchronized and then treated with TGF- β 1 (1 ng/ml or 10 ng/ml) in **A**: 0 FBS- and **B**: 10% FBS-containing DMEM for 24 h. mRNA expression of STIM1 and Orai1 was analyzed with real-time PCR. Shown are means $\pm$ SE of 4 independent experiments from 3 different animals. Δ Ct represents the difference between the threshold cycle of STIM1 or Orai1 and that of β -actin. * p < .05 vs. control.

in TGF- β 1-treated ASMCs, since the amplitude of Ca^{2+} entry depends on the degree of Ca^{2+} store depletion (27). However, this may not be the case in the presence of antioxidant α -tocopherol. As shown in Figure 4, α -tocopherol treatment decreased TPG-induced Ca^{2+} release in control and TGF- β 1-treated ASMCs, but the amplitude of subsequent SOCE was not changed by this antioxidant. Since TGF- β 1 also increased the expression of STIM1 and Orai1, it is necessary to investigate the expression levels of both genes after treatment with α -tocopherol.

We also examined the possible role of IK_{Ca} in mediating the effect of TGF- β 1 on SOCE. Previous studies demonstrated that TGF- β 1 regulates both the expression and the gating properties of IK_{Ca} channels (15, 28), whose activation was initiated by an increase of $[\text{Ca}^{2+}]_i$ and will sustain the Ca^{2+} influx through SOC channels, as we have demonstrated on human macrophages (29). We showed that both the peak amplitude and the decay rate of plateau Ca^{2+} were not affected by IK_{Ca} blocker CTX, suggesting that IK_{Ca} should not be involved in the modulation of SOCE by TGF- β 1. Glucocorticoids were the mainstay of current asthma therapy. Unexpectedly, we did not find any effect of DXM on TGF- β 1-promoted basal $[\text{Ca}^{2+}]_i$, TPG-induced Ca^{2+} release, and SOCE. This is different from what we observed in IL-13-promoted Ca^{2+} signaling, which was sensitive to DXM treatment (19).

Both STIM1 and Orai1 are essential to the functional activity of SOC channels of rat ASMCs, and the mRNA level of Orai1, but not STIM1, was significantly increased

in proliferating ASMCs, as we showed previously (13). A latest study also showed that the expression of STIM1 and Orai1 was increased in ASMCs from asthmatic mice (30). Interestingly, the mRNA expression of STIM1 and Orai1 in ASMCs was significantly increased by TGF- β 1 (10 ng/ml) in the presence of 10% FBS. In FBS-free medium, TGF- β 1 had negligible effect on the expression of both genes, indicating a necessary synergic effect of other growth factors to initiate the transcriptional regulation of both genes by TGF- β 1 (31). We found that TGF- β 1 had significantly increased the proliferation of ASMCs under stimulation with 2.5% FBS. This is consistent with a previous study which showed that TGF- β 1 stimulated ASM cell growth in the presence of lower concentration of serum (7). The upregulated expression of both STIM1 and Orai1 may partly contribute to the increased SOCE induced by TGF- β 1. In addition, the effect of TGF- β 1 on the expression of STIM1 and Orai1 may also take place at protein level or post-transcriptional level and should be elucidated later.

In the present study, we also clearly demonstrated that SOCE was involved in ASMC proliferation facilitated by TGF- β 1. This effect of TGF- β 1 was prominent only under stimulation with 2.5% FBS but not 1% FBS or 10% FBS (data not shown), similar to other studies which showed that lower concentration of FBS is optimal for detecting the effect of TGF- β 1 on the proliferation (7, 32). The cause for the requirement of lower dose serum in revealing the effect of TGF- β 1 on the proliferation of ASMCs is not clarified, but a permissive role of other growth factors has been suggested to cause ASMC proliferation (31).

Non-specific Ca^{2+} channel blockers Ni^{2+} , La^{3+} , and SKF-96365 were potent inhibitors of SOC channels in ASMCs (19). BTP-2 was a relatively selective SOC blocker. It has been shown that BTP-2 could inhibit the development of eosinophilic airway inflammation and airway hyperresponsiveness in ovalbumin (OVA)-challenged guinea pigs (33, 34). TGF- β 1-promoted ASMC proliferation was partly inhibited by Ni^{2+} , SKF-96365, and BTP-2 (Figure 6B–D), suggesting that SOC channels are involved in the proliferation promoted by TGF- β 1. SKF-96365 also inhibits the activity of receptor-operated Ca^{2+} channels (ROC), therefore, Ca^{2+} entry through ROC (ROCE) may also contribute to TGF- β 1-promoted ASMC proliferation. La^{3+} had no inhibitory effect on the proliferation of ASMC (Figure 6B and C). We speculate that this may be due to its potent inhibiting effect on the activity of plasma membrane Ca^{2+} -ATPase (PMCA) (35), whose dysfunction will impair Ca^{2+} extrusion.

A recent study demonstrated that TGF- β 1 was associated with increased airway oxidant burden in asthma patients, suggesting that oxidant stress might mediate the effects of TGF- β 1 and promotes airway remodeling (36). This is consistent with our results that TGF- β 1 promoted proliferation of ASMCs. Antioxidant α -tocopherol inhibited serum-induced proliferation of A7r5 vascular smooth muscle cells (37). Our results showed that α -tocopherol had no effect on serum-induced proliferation of ASMCs (Figure 6C). We speculate that this difference may be due to distinct tissue

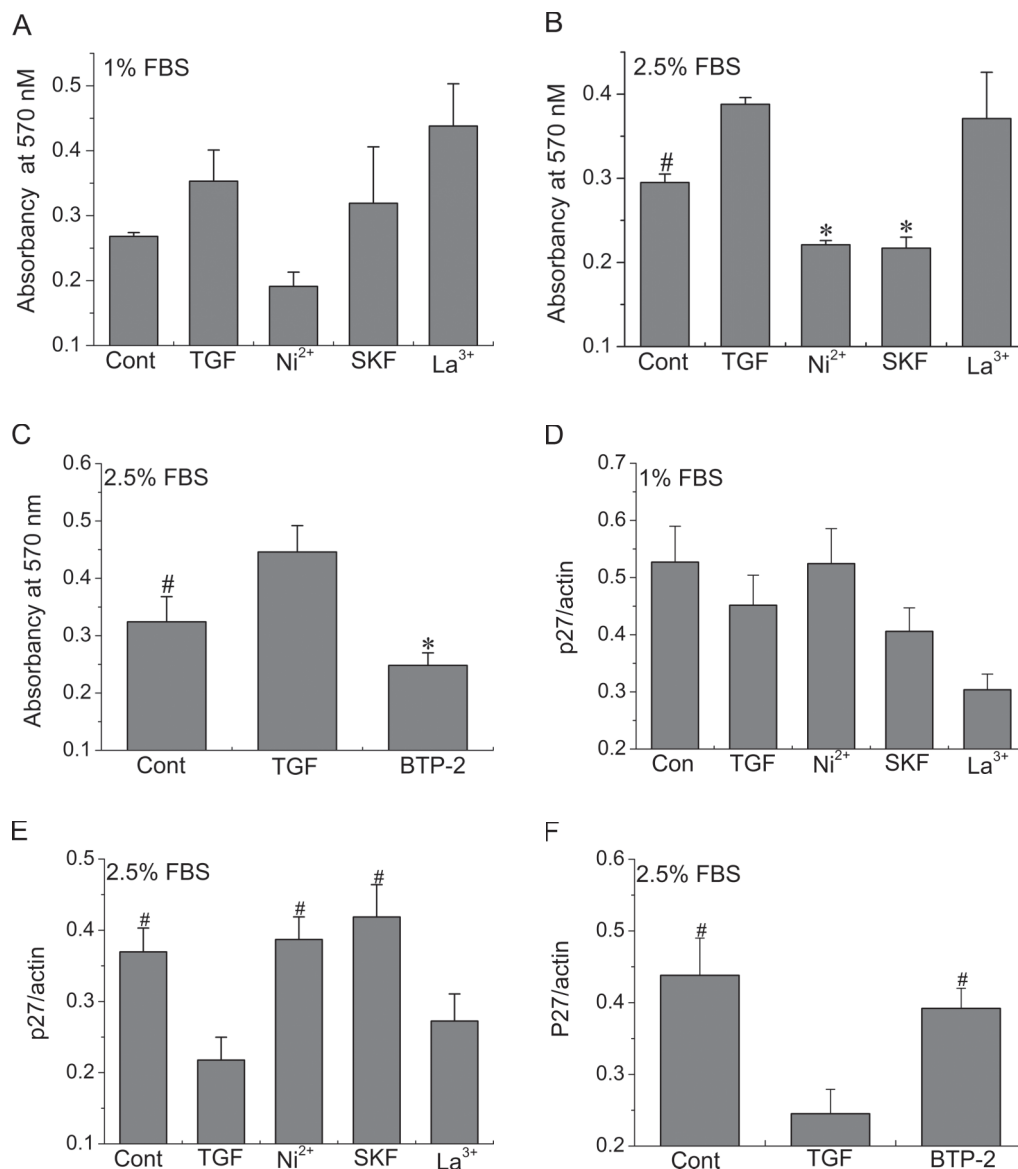


FIGURE 6.—Effect of TGF- β 1 on the proliferation of rat ASMCs. The proliferation rates of ASMCs were determined by MTT and P27 expression assay as described in Methods. ASMCs grown in 96-well plates were synchronized and then stimulated with 1% FBS or 2.5% FBS-containing DMEM for 24 h, the cells were then treated with PBS as control, TGF- β 1 (TGF, 10 ng/ml), TGF- β 1 together with Ca²⁺ channel blockers SKF-96365 (SKF, 10 μ M), NiCl₂ (Ni²⁺, 100 μ M), LaCl₃ (La³⁺, 100 μ M) and BTP-2 (100 nM). **A and D**: Ca²⁺ channels blockers SKF-96365, NiCl₂ and LaCl₃ on TGF- β 1-promoted ASMC proliferation stimulated with 1% FBS. **B and E**: Ca²⁺ channels blockers SKF-96365, NiCl₂ and LaCl₃ on TGF- β 1-promoted ASMC proliferation stimulated with 2.5% FBS. **C and F**: Ca²⁺ channels blocker BTP-2 on TGF- β 1-promoted ASMC proliferation stimulated with 2.5% FBS. Data are representative of three independent experiments. #*p* < .05, **p* < .01, compared to TGF group.

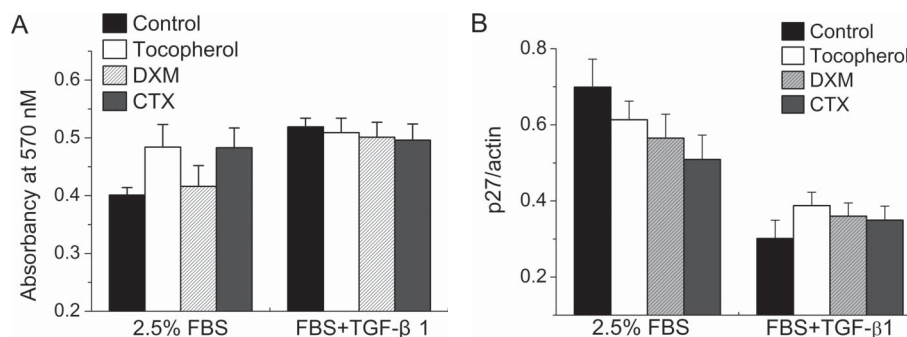


FIGURE 7.— α -tocopherol (100 μ M), dexamethasone (DXM, 10⁻⁷ M) and IK_{Ca} blocker charybdotoxin (CTX, 100 nM) had no effect on 2.5% FBS-stimulated and TGF- β 1-promoted ASMC proliferation. The proliferation rate of ASMCs was determined with MTT assay (**A**) and P27 expression assay (**B**). Data are representative of three independent experiments.

properties. DXM did not affect the proliferation of both control and TGF- β 1-treated ASMCs, similar to our previous study on IL-13-treated ASMCs (19).

In conclusion, TGF- β 1 promotes the proliferation of rat ASMCs by increasing activity of SOCE and expression of STIM1 and/or Orai1.

ACKNOWLEDGMENTS

We thank B.Sc Bo-quan Pan (Biomedical Research Center, Wuhan University Medical College) for the technical support.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. This work was supported by NSFC of China (81072684 to Gao YD).

REFERENCES

- Panettieri RA, Jr., Kotlikoff MI, Gerthoffer WT, Hershenson MB, Woodruff PG, Hall IP, Banks-Schlegel S. Airway smooth muscle in bronchial tone, inflammation, and remodeling: Basic knowledge to clinical relevance. *Am J Respir Crit Care Med* 2008; 177:248–252.
- Al-Muhsen S, Johnson JR, Hamid Q. Remodeling in asthma. *J Allergy Clin Immunol* 2011; 128:451–462; quiz 63–64.
- Minshall EM, Leung DY, Martin RJ, Song YL, Cameron L, Ernst P, Hamid Q. Eosinophil-associated TGF- β 1 mRNA expression and airways fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 1997; 17:326–333.
- Ohno I, Nitta Y, Yamauchi K, Hoshi H, Honma M, Woolley K, O'Byrne P, Tamura G, Jordana M, Shirato K. Transforming growth factor beta 1 (TGF beta 1) gene expression by eosinophils in asthmatic airway inflammation. *Am J Respir Cell Mol Biol* 1996; 15:404–409.
- Perng DW, Wu YC, Chang KT, Wu MT, Chiou YC, Su KC, Perng RP, Lee YC. Leukotriene C4 induces TGF- β 1 production in airway epithelium via p38 kinase pathway. *Am J Respir Cell Mol Biol* 2006; 34:101–107.
- Woodman L, Siddiqui S, Cruse G, Sutcliffe A, Saunders R, Kaur D, Bradding P, Brightling C. Mast cells promote airway smooth muscle cell differentiation via autocrine up-regulation of TGF- β 1. *J Immunol* 2008; 181:5001–5007.
- Xie S, Sukkar MB, Issa R, Khorasani NM, Chung KF. Mechanisms of induction of airway smooth muscle hyperplasia by transforming growth factor-beta. *Am J Physiol Lung Cell Mol Physiol* 2007; 293: L245–L253.
- Sturrock A, Huecksteadt TP, Norman K, Sanders K, Murphy TM, Chitano P, Wilson K, Hoidal JR, Kennedy TP. Nox4 mediates TGF- β 1-induced retinoblastoma protein phosphorylation, proliferation, and hypertrophy in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2007; 292:L1543–L1555.
- Cohen MD, Ciocca V, Panettieri RA, Jr. TGF- β 1 modulates human airway smooth-muscle cell proliferation induced by mitogens. *Am J Respir Cell Mol Biol* 1997; 16:85–90.
- Ito I, Fixman ED, Asai K, Yoshida M, Gounni AS, Martin JG, Hamid Q. Platelet-derived growth factor and transforming growth factor-beta modulate the expression of matrix metalloproteinases and migratory function of human airway smooth muscle cells. *Clin Exp Allergy* 2009; 39:1370–1380.
- Baarsma HA, Menzen MH, Halayko AJ, Meurs H, Kerstjens HA, Gosens R. beta-Catenin signaling is required for TGF- β 1-induced extracellular matrix production by airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2011; 301:L956–L965.
- Perez-Zoghbi JF, Karner C, Ito S, Shepherd M, Alrashdan Y, Sanderson MJ. Ion channel regulation of intracellular calcium and airway smooth muscle function. *Pulm Pharmacol Ther* 2009; 22:388–397.
- Zou JJ, Gao YD, Geng S, Yang J. Role of STIM1/Orai1-mediated store-operated Ca(2+)(+) entry in airway smooth muscle cell proliferation. *J Appl Physiol* 2011; 110:1256–1263.
- Kim JH, Jain D, Tliba O, Yang B, Jester WF, Jr., Panettieri RA, Jr., Amrani Y, Pure E. TGF-beta potentiates airway smooth muscle responsiveness to bradykinin. *Am J Physiol Lung Cell Mol Physiol* 2005; 289: L511–L520.
- Shepherd MC, Duffy SM, Harris T, Cruse G, Schuliga M, Brightling CE, Neylon CB, Bradding P, Stewart AG. KCa3.1 Ca²⁺ activated K⁺ channels regulate human airway smooth muscle proliferation. *Am J Respir Cell Mol Biol* 2007; 37:525–531.
- Junn E, Lee KN, Ju HR, Han SH, Im JY, Kang HS, Lee TH, Bae YS, Ha KS, Lee ZW, Rhee SG, Choi I. Requirement of hydrogen peroxide generation in TGF-beta 1 signal transduction in human lung fibroblast cells: Involvement of hydrogen peroxide and Ca²⁺ in TGF-beta 1-induced IL-6 expression. *J Immunol* 2000; 165:2190–2197.
- Sharma K, Deelman L, Madesh M, Kurz B, Ciccone E, Siva S, Hu T, Zhu Y, Wang L, Henning R, Ma X, Hajnoczky G. Involvement of transforming growth factor-beta in regulation of calcium transients in diabetic vascular smooth muscle cells. *Am J Physiol Renal Physiol* 2003; 285: F1258–F1270.
- Neylon CB, Bryant SM, Little PJ, Bobik A. Transforming growth factor-beta 1 regulates the expression of ryanodine-sensitive Ca²⁺ oscillations in cardiac myocytes. *Biochem Biophys Res Commun* 1994; 204:678–684.
- Gao YD, Zou JJ, Zheng JW, Shang M, Chen X, Geng S, Yang J. Promoting effects of IL-13 on Ca²⁺ release and store-operated Ca²⁺ entry in airway smooth muscle cells. *Pulm Pharmacol Ther* 2010; 23:182–189.
- Michaeloudes C, Sukkar MB, Khorasani NM, Bhavsar PK, Chung KF. TGF-beta regulates Nox4, MnSOD and catalase expression, and IL-6 release in airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2011; 300:L295–L304.
- Grupe M, Myers G, Penner R, Fleig A. Activation of store-operated I (CRAC) by hydrogen peroxide. *Cell Calcium* 2010; 48:1–9.
- Lin MJ, Yang XR, Cao YN, Sham JS. Hydrogen peroxide-induced Ca²⁺ mobilization in pulmonary arterial smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2007; 292:L1598–L1608.
- Mabalarajan U, Aich J, Leishangthem GD, Sharma SK, Dinda AK, Ghosh B. Effects of vitamin E on mitochondrial dysfunction and asthma features in an experimental allergic murine model. *J Appl Physiol* 2009; 107:1285–1292.
- Goldsmith AM, Bentley JK, Zhou L, Jia Y, Bitar KN, Fingar DC, Hershenson MB. Transforming growth factor-beta induces airway smooth muscle hypertrophy. *Am J Respir Cell Mol Biol* 2006; 34:247–254.
- Bottoms SE, Howell JE, Reinhardt AK, Evans IC, McAnulty RJ. Tgf-Beta isoform specific regulation of airway inflammation and remodelling in a murine model of asthma. *PLoS One* 2010; 5:e9674.
- Kimura H, Takemura H, Imoto K, Ohshika H, Mochizuki Y. Upregulation of expression of sarcoplasmic reticulum by TGF-beta 1 in cultured rat cardiac myocytes. *Am J Physiol* 1997; 272: H2639–44.
- Parekh AB, Putney JW, Jr. Store-operated calcium channels. *Physiol Rev* 2005; 85:757–810.
- Lhuillier L, Dryer SE. TGF-beta1 regulates the gating properties of intermediate-conductance KCa channels in developing parasympathetic neurons. *J Neurophysiol* 1999; 82:1627–1631.
- Gao YD, Hanley PJ, Rinne S, Zuzarte M, Daut J. Calcium-activated K (+) channel (K(Ca)3.1) activity during Ca(2+) store depletion and store-operated Ca(2+) entry in human macrophages. *Cell Calcium* 2010; 48:19–27.
- Spinelli AM, Gonzalez-Cobos JC, Zhang X, Motiani RK, Rowan S, Zhang W, Garrett J, Vincent PA, Matrougui K, Singer HA, Trebak M. Airway smooth muscle STIM1 and Orai1 are upregulated in asthmatic mice and mediate PDGF-activated SOCE, CRAC currents, proliferation, and migration. *Pflugers Arch* 2012; 464:481–492.

31. Bosse Y, Stankova J, Rola-Pleszczynski M. Transforming growth factor-beta1 in asthmatic airway smooth muscle enlargement: is fibroblast growth factor-2 required? *Clin Exp Allergy* 2010; 40:710–724.
32. Chen G, Khalil N. TGF-beta1 increases proliferation of airway smooth muscle cells by phosphorylation of map kinases. *Respir Res* 2006; 7:2.
33. Ohga K, Takezawa R, Yoshino T, Yamada T, Shimizu Y, Ishikawa J. The suppressive effects of YM-58483/BTP-2, a store-operated Ca^{2+} entry blocker, on inflammatory mediator release in vitro and airway responses in vivo. *Pulm Pharmacol Ther* 2008; 21:360–399.
34. Ohga K, Takezawa R, Arakida Y, Shimizu Y, Ishikawa J. Characterization of YM-58483/BTP2, a novel store-operated Ca^{2+} entry blocker, on T cell-mediated immune responses in vivo. *Int Immunopharmacol* 2008; 8:1787–1792.
35. Di Capite J, Ng SW, Parekh AB. Decoding of cytoplasmic Ca^{2+} oscillations through the spatial signature drives gene expression. *Curr Biol* 2009; 19:853–858.
36. Brown SD, Baxter KM, Stephenson ST, Esper AM, Brown LA, Fitzpatrick AM. Airway TGF-beta1 and oxidant stress in children with severe asthma: association with airflow limitation. *J Allergy Clin Immunol* 2012; 129:388–96, 96 e1–8.
37. Chatelain E, Boscoboinik DO, Bartoli GM, Kagan VE, Gey FK, Packer L, Azzi A. Inhibition of smooth muscle cell proliferation and protein kinase C activity by tocopherols and tocotrienols. *Biochim Biophys Acta* 1993; 1176:83–89.