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Upregulation of Transient Receptor Potential Canonical Channels Contributes to Endotoxin-Induced Pulmonary Arterial Stenosis

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEFG 1,2 **Gui-Lan Chen**
ABCDEFG 3 **Hongni Jiang**
ADE 1 **Fangdong Zou**

1 Ministry of Education Key Laboratory of Bio-Resources and Eco-Environment, College of Life Sciences, Sichuan University, Chengdu, Sichuan, P.R. China
2 Key Laboratory of Medical Electrophysiology, Ministry of Education, and Institute of Cardiovascular Research, Sichuan Medical University, Luzhou, Sichuan, P.R. China
3 Department of Pulmonary Medicine, Zhongshan Hospital, Fudan University School of Medicine, Shanghai, P.R. China

Corresponding Authors: Fangdong Zou, e-mail: fundzou@scu.edu.cn; Hongni Jiang, e-mail: jhn2001@sina.com

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Background: Septic shock is a pathologic condition caused by endotoxin-producing bacteria, and often associated with severe pulmonary hypertension. Inflammation is a major systemic response to endotoxin; however, it is unknown whether endotoxin has a direct impact on pulmonary arteries that contributes to pathogenesis of pulmonary hypertension.

Material/Methods: Rat pulmonary arteries and primary pulmonary arterial smooth muscle cells (PASMCs) were cultured *in vitro* and treated with lipopolysaccharide (LPS) and blockers of transient receptor potential canonical (TRPC) channels. Neointimal growth and arterial stenosis were observed on cryosections of cultured pulmonary arteries. Proliferation of PASMCs was examined by a WST-1 (water-soluble tetrazolium salt) assay. Expression of TRPC genes in pulmonary arteries and PASMCs were detected and quantified by real-time polymerase chain reaction and Western blotting.

Results: LPS significantly induced neointimal growth and stenosis of pulmonary arteries and promoted proliferation of PASMCs. TRPC channel blockers 2-aminoethoxydiphenyl borate and SKF-96365 inhibited LPS-induced remodeling of pulmonary arteries and PASMC proliferation. Expression of TRPC1/3/4/6 was detected in pulmonary arteries and PASMCs. LPS treatment dramatically increased the expression of TRPC3 and TRPC4 at both messenger RNA and protein levels.

Conclusions: LPS stimulates stenosis of pulmonary arteries through enhancement of TRPC-mediated Ca^{2+} entry into PASMCs, which is caused by upregulation of TRPC3 and TRPC4 channels.

MeSH Keywords: **Endotoxins • Gene Expression • Pulmonary Artery • TRPC Cation Channels**

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Background

Septic shock is characterized by vascular dysfunction, coagulation disorder, multiple organ failure, and finally death. Bacterial infection and consequent endotoxin (lipopolysaccharide [LPS]) exposure is the original cause of sepsis [1], and the severe inflammatory response to LPS is considered an important mediator of septic shock [2]. Vascular dysfunction, including systemic hypotension and pulmonary artery hypertension (PAH), is a life-threatening condition in septic shock [3,4]. LPS-induced inflammation has been found to affect vascular endothelial function of pulmonary hypertension [5]. However, it is unknown whether LPS has a direct impact on the structure and function of pulmonary arteries.

Transient receptor potential canonical (TRPC) channels are a class of nonselective Ca^{2+}-permeable channels consisting of 7 members (TRPC1-7). TRPC2 is different from the other members because it is a pseudogene in humans [6]. TRPC channels are activated by stimulation of G protein-coupled receptors (GPCRs), such as endothelin [7], angiotensin [8], and muscarinic acetylcholine receptors [9], and thus are called receptor-operated channels (ROCs). Studies on TRPC4-knockout rats and TRPC1/6-knockout mice have shown that deficiency of these channels suppressed the development of PAH [10–12]. TRPC channels are essential for many Ca^{2+}-dependent functions of pulmonary arterial smooth muscle cells (PASMCs) [13], such as cell proliferation [14] and contraction [15]. In this study, we investigated the involvement of TRPC channels in LPS-induced pathophysiologic changes in pulmonary arteries and PASMCs. To exclude the impact of immunologic response to LPS, we used *in vitro* culture models to observe the direct effect of LPS on pulmonary arteries and PASMCs.

Material and Methods

Drugs and reagents

General salts, collagenase I, papain, LPS, SKF-96365, and 2-aminoethoxydiphenyl borate (2-APB) were purchased from Sigma-Aldrich (USA).

Neointimal growth assay of pulmonary arteries

Male Sprague Dawley rats weighing 150 to 200 g were used in experiments, in accordance with the local guidelines for the care and use of laboratory animals and approved by the Local Committee of Animal Use. The animals were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg). Pulmonary arteries with diameters approximately 0.5 mm were dissected out from both lungs, cut into segments about 2 mm long, and soaked in HEPES-buffered physiologic salt solution (HBSS) containing NaCl 130 mM, KCl 5 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 1.5 mM, HEPES 10 mM, and glucose 10 mM, with pH adjusted to 7.4 with 5 M NaOH. The arterial segments were placed in 35-mm culture dishes with Dulbecco's modified eagle medium (DMEM)/F-12 medium containing 20% fetal bovine serum (FBS; HyClone, USA), 100 units/mL penicillin, and 100 mg/mL streptomycin. The medium was changed every 3 days without touching the vessels. After 27 days of culture the arterial segments were embedded in Cryomatrix resin (Thermo Scientific, UK). Frozen sections with 20 μm thickness were made in a cryostat and observed under 10x magnification. The images were captured by a digital camera and measured with NIS-Elements software (Nikon, Japan).

Isolation and proliferation assay of PASMCs

PASMCs were isolated as previously reported with a minor modification [16]. Briefly, rats were sacrificed and pulmonary arteries were isolated in ice-cold HBSS. After cleaning the adventitia and intima, the pulmonary artery was minced and digested at 37°C for 15 to 17 min in reduced-Ca^{2+} HBSS (20 μM Ca^{2+}) containing collagenase (type I, 2 mg/mL), papain (1.5 mg/mL), bovine serum albumin (2 mg/mL), and DTT (1 mM). Single smooth muscle cells were dispersed by gentle trituration in cold reduced-Ca^{2+} HBSS, and then transferred into DMEM/F-12 medium containing 10% FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin. PASMCs at passage 2 were seeded into 96-well plates for cell proliferation assay with WST-1 reagent (Roche, USA) according to the manufacturer's instruction.

Real-time PCR

Total RNA was extracted from cultured pulmonary arteries and PASMCs using the TRIzol reagent method according to the manufacturer's instructions (Invitrogen, USA). Oligo(dT)-primed first-strand cDNA synthesis was performed using avian myeloblastosis virus reverse transcriptase (Promega, USA) with 2 μg RNA as template in a total volume of 20 μL. The cDNA was diluted 30 times and then used for real-time PCR with Taq DNA polymerase (Promega, USA) and SYBR Green (Fluka, Germany). The primer sequences are listed in Table 1. β-actin was used as the internal control.

Western blotting

Tissues or cells were homogenized and lysed in radioimmunoprecipitation assay buffer (Beyotime, Jiangsu, China), and then proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel before transferring onto nitrocellulose membrane. The blot was incubated with primary antibody (rabbit polyclonal anti-TRPC3 from Sangon, Shanghai, China, and anti-TRPC4 from Alomone Labs, Jerusalem, Israel) overnight at 4°C, washed with Tris-buffered

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Table 1. PCR primers for rat genes studied.

Gene	Primer sequences (5'-3')	Annealing temperature (°C)	Product size (bp)
TRPC1	GGATTATTGGGATGATTTGGT GTGAGCCACCACTTTGAGG	55	143
TRPC3	ACATCACCGTTATTGACTACCC GCACTCAGACCACATCATCC	55	113
TRPC4	ACCATCGTGGAGTGGATGA TGTCGCCAGATACAAGGAGT	55	147
TRPC5	CAACTGTCGTGGAATGGATG CCAGGTAGAGGGAGTTCATTG	55	144
TRPC6	TCCGAATCTCAGCCGTTT ATGGTCTGCTGCCGTAAAC	55	129
TRPC7	CGCCTACCTGTCCCTATCC CACGCCCACCACAAAGTC	55	148
β-actin	TGAACCCTAAGGCCAACC AGAGGCATACAGGGACAACA	55	107

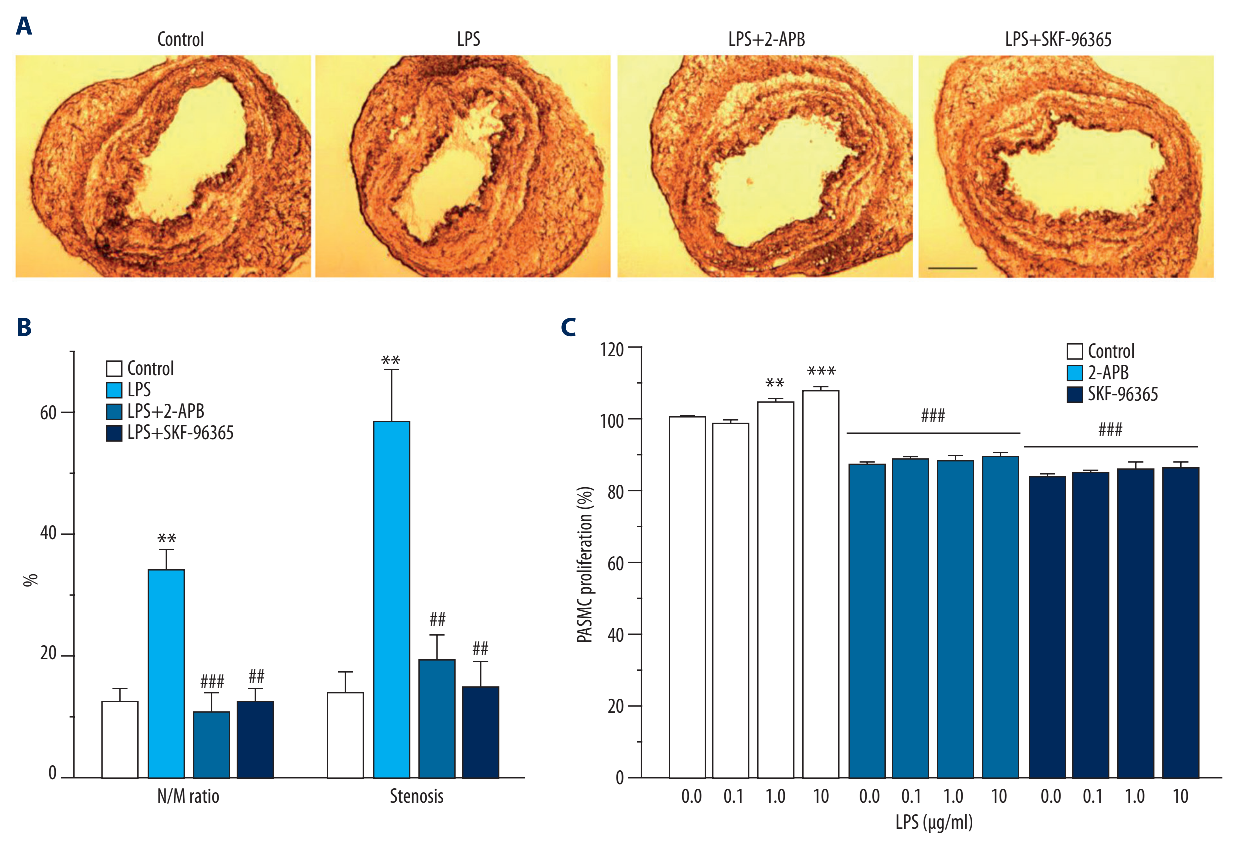

Figure 1. Effects of lipopolysaccharide (LPS) and transient receptor potential canonical (TRPC) blockers on neointima formation of rat pulmonary arteries and proliferation of pulmonary arterial smooth muscle cells (PASMCs). (**A**) Frozen sections of pulmonary arteries after culturing for 27 days without/with LPS (10 µg/mL), 2-aminoethoxydiphenyl borate (2-APB; 100 µM), and SKF-96365 (100 µM). Scale bar: 0.1 mm. (**B**) Ratio between the areas of neointima and media and percentage of decreased luminal area (stenosis) in the arterial sections. ** *P*<0.01 *vs.* control group; ## *P*<0.01; ### *P*<0.001 *vs.* LPS group; *n*=5 in each group. (**C**) Result of WST assay on PASMCs. 2-APB and SKF-96365 at 100 µM were used. ** *P*<0.01; *** *P*<0.001 *vs.* LPS 0 control; ### *P*<0.001 *vs.* corresponding columns in the control group; *n*=8 in each column.

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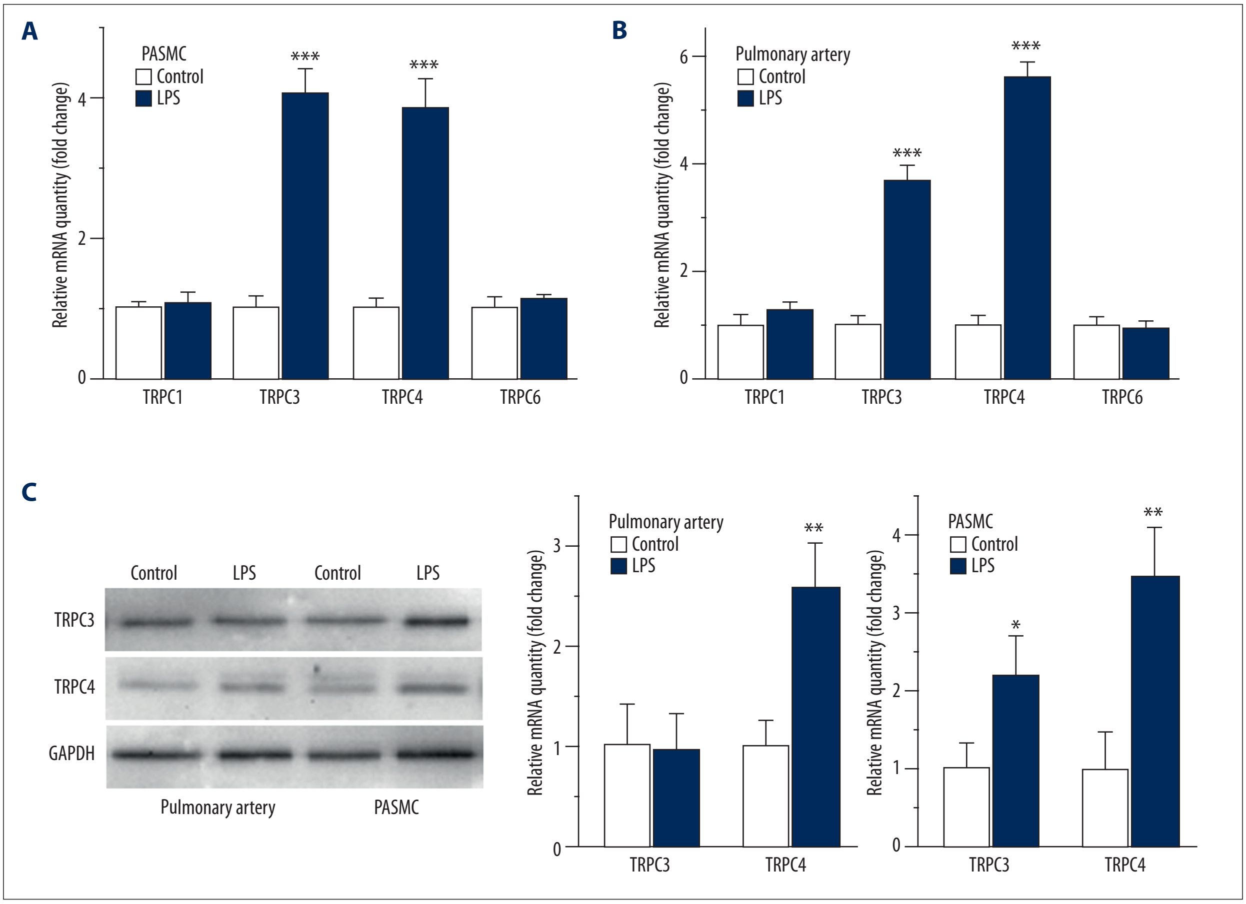

Figure 2. Lipopolysaccharide (LPS) upregulated the expression of transient receptor potential canonical 3 (TRPC3) and 4 (TRPC4) channels in rat pulmonary arteries and proliferation of pulmonary arterial smooth muscle cells (PASMCs). The expression levels of TRPC1/3/4/6 messenger RNA (mRNA) in cultured pulmonary arteries (**A**) and PASMCs (**B**) are shown. β-actin was used for normalization of mRNA. (**C**) Protein expression levels of TRPC3 and TRPC4. GAPDH was used as an internal control. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; $n=6$ (mRNA) and $n=3$ (protein) in each group.

saline and Tween 20, and then incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, Sangon, Shanghai, China). Rabbit anti-GAPDH (anti-glyceraldehyde 3-phosphate dehydrogenase; Santa Cruz Biotech, USA) was used as an internal standard for protein quantification. Visualization was carried out using enhanced chemiluminescence detection reagents (Engreen, Beijing, China). Images were captured by a gel documentation system and the band density was analyzed using Quantity One (Bio-Rad, Hercules, USA).

Statistics

All values are expressed as plus or minus standard error of the mean. Unpaired *t* test was used to assess the statistical difference between the 2 groups, and a 1-way analysis of variance was used in comparison of more than 2 groups. $P<0.05$ was considered significant.

Results

LPS promoted neointimal growth of pulmonary arteries and proliferation of PASMCs

After 27 days of culture, irregular thickening of the intima of the pulmonary arterial segments was observed (Figure 1A). The neointimal area of LPS-treated arteries (0.068±0.009 mm^2) was significantly larger ($P<0.001$) than that in the control group (0.025±0.008 mm^2), whereas the treatment with 2 TRPC channel blockers – 2-APB (0.024±0.012 mm^2) and SKF-96365 (0.025±0.011 mm^2) – inhibited the neointimal growth induced by LPS ($P<0.001$ for both). Consistent with these observations, the ratio between the neointimal and medial areas also showed dramatic increase in the LPS group, while 2-APB and SKF-96365 abolished the effect of LPS (Figure 1B). Luminal area in the arteries was reduced by LPS by 58%, which is much higher than in the control (14%), 2-APB (19%), and SKF-96365 (15%) groups (Figure 1B). Because the neointimal formation of blood vessels is largely attributed to the proliferation of smooth muscle cells, we assessed whether LPS can stimulate the growth of isolated PASMCs using a WST-1 assay. As shown in Figure 1C, LPS at 1 and 10 µg/mL significantly promoted the proliferation of PASMCs after 24 h of incubation, which was potently inhibited by 2-APB and SKF-96365, suggesting the involvement of TRPC channels in LPS-triggered cellular processes.

LPS upregulated the expression of TRPC3 and TRPC4 in pulmonary arteries and PASMCs

We used real-time PCR to detect the expression of TRPC channels including TRPC1, TRPC3, TRPC4, TRPC5, TRPC6, and TRPC7 in cultured pulmonary arteries and PASMCs. Among these genes, TRPC5 and TRPC7 were not detected. Quantitative real-time PCR results showed that LPS dramatically increased messenger RNA (mRNA) expression of TRPC3 and TRPC4 in pulmonary arteries, whereas no change was observed for TRPC1 and TRPC6 (Figure 2A). Similar results were found for TRPC genes expressed in PASMCs (Figure 2B). To confirm the upregulation of TRPC3 and TRPC4 by LPS, we analyzed the protein expression of TRPC3 and TRPC4 using Western blotting. In pulmonary arteries, LPS only significantly increased the expression of TRPC4, while both TRPC3 and TRPC4 were robustly upregulated in PASMCs (Figure 2C).

Discussion

Vascular smooth muscle cells undergo phenotypic modulation from a contractile phenotype to a proliferative phenotype during cell culture [17,18]. Our study was conducted on cultured pulmonary arteries and freshly isolated PASMCs. This experimental system avoided LPS-induced extensive immunologic interference *in vivo* and could show the direct effect of LPS on pulmonary arteries and PASMCs. It had been established that LPS-induced inflammation could increase neointimal formation in rabbit aorta and iliac artery after vascular injury [19]. However, it was unknown whether LPS had a direct action on the growth of vascular smooth muscle cells. Our results showed that LPS treatment promoted stenosis of pulmonary arteries and proliferation of PASMCs. These effects are abolished by blockers of TRPC channels, which are essential for the proliferation of vascular myocytes [20]. We further demonstrated that the LPS-stimulated neointimal growth and proliferation of PASMCs should be attributed to increased expression of TRPC3 and TRPC4 channels. Upregulation of TRPC channels could result in more Ca^{2+} flow into the cells upon activation of many GPCRs. The activities of Ca^{2+}-dependent enzymes, such as calcineurin, which controls the phosphorylative state of the nuclear factor of activated T cell (NFAT) [21,22], were therefore enhanced. Dephosphorylated NFAT then enters the nucleus and induces the expression of massive genes related to cell proliferation [23]. The expression of these genes promotes the cells to a proliferative state. When more PASMCs accumulate at the intima of the pulmonary artery, the internal arterial diameter becomes smaller and thus promotes pulmonary hypertension. This could be a novel pathologic mechanism of pulmonary arterial hypertension in septic shock.

Conclusions

Our results show that TRPC channels in PASMCs are crucial for LPS-induced stenosis of pulmonary arteries. Application of TRPC blockers in animal models of septic shock-induced pulmonary hypertension is suggested for further studies.

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