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

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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 Ca²⁺ 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) according to the manufacturer’s instruction method. The primer sequences were listed in Table 1. 18s-rRNA was used as the internal control.

Western blotting

Tissues or cells were homogenized and lysed in radioimmuno-precipitation 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
Table 1. PCR primers for rat genes studied.

| Gene | Primer sequences (5’-3’) | Annealing temperature (°C) | Product size (bp) |
|------|--------------------------|----------------------------|------------------|
| TRPC1 | GGATTATTGGGATGATTTGGT GTGAGCCACCATTTTGGG | 55 | 143 |
| TRPC3 | ACATACGTTAATTGACTACCC GCACGTAAGACACATCC | 55 | 113 |
| TRPC4 | ACCACTGGAAGTTGATGTA TGTCGCAGATACAAAGAGT | 55 | 147 |
| TRPC5 | CAACCTGTCGATGATGATC CCACTGAGGAGGATTCATTG | 55 | 144 |
| TRPC6 | TCCGATCTCAGCGCTTI ATGGTCGTCGGCCTAAC | 55 | 129 |
| TRPC7 | CGCCTACCTGCTCCTATCC CACGCCCACCAAAAGTC | 55 | 148 |
| β-actin | TGAACCCAAGGCAAACC AGAGCCATACAGGGACAAA | 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.
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.

**References:**

1. Opal SM, Scannon PJ, Vincent JL et al: Relationship between plasma levels of lipopolysaccharide (LPS) and LPS-binding protein in patients with severe sepsis and septic shock. J Infect Dis, 1999; 180(5): 1584–89
2. Dellingger RP, Levy MM, Rhodes A et al: Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock. 2012. Crit Care Med, 2013; 41(2): 580–637
3. Matsuda N, Hattori Y: Vascular biology in sepsis: Pathophysiological and therapeutic significance of vascular dysfunction. J Smooth Muscle Res, 2007; 43(4): 117–37
4. Patel R, Aronow WS, Patel L et al: Treatment of pulmonary hypertension. Med Sci Monit, 2012; 18(4): RA31–39
5. Le Hirress M, Tu L, Ricard N et al: Proinflammatory signature of the dysfunctional endothelium in pulmonary hypertension. Role of the macrophage migration inhibitory factor/CD74 complex. Am J Respir Crit Care Med, 2015; 192(8): 983–97
6. Vannier B, Peyton M, Boulay G et al: Mouse trp2, the homologue of the human trpc2 pseudogene, encodes mTrp2, a store depletion-activated capacitative Ca^{2+} entry channel. Proc Natl Acad Sci USA, 1999; 96(5): 2060–64
7. Shi J, Ju M, Large WA, Albert AP: Pharmacological profile of phosphatidylinositol 3-kinases and related phosphatidylinositol 3-kinases mediating endothelin(A) receptor-operated native TRPC channels in rabbit coronary artery myocytes. Br J Pharmacol, 2012; 166(7): 2161–75
8. Evans IF, Lee JH, Ragolia L: Ang-II-induced Ca(2+) influx is mediated by the 1A/5 subgroup of the transient receptor potential proteins in cultured aortic smooth muscle cells from diabetic Goto-Kakizaki rats. Mol Cell Endocrinol, 2009; 302(1): 49–57
9. Boulay G, Zhu X, Peyton M et al: Cloning and expression of a novel mammalian homolog of Drosophila transient receptor potential (TRP) involved in calcium entry secondary to activation of receptors coupled by the Gq class of G protein. J Biol Chem, 1997; 272(47): 29672–80
10. Alzoubi A, Almalouf P, Toba M et al: TRPC4 inactivation confers a survival benefit in severe pulmonary arterial hypertension. Am J Pathol, 2013; 183(6): 1779–88
11. Xia Y, Yang XR, Fu Z et al: Classical transient receptor potential channel 1 in hypoxia-induced pulmonary hypertension. Am J Respir Crit Care Med, 2013; 188(2): 1451–59
12. Malczyk M, Veith C, Fuchs B et al: Classical transient receptor potential channel 1 promotes pulmonary hypertension. Am J Respir Crit Care Med, 2013; 188(12): 1451–59
13. Guibert C, Ducret T, Savineau JP: Expression and physiological roles of TRP channels in smooth muscle cells. Adv Exp Med Biol, 2011; 704: 687–706
14. Sweeney M, Yu Y, Platsoshyn O et al: Inhibition of endogenous TRP1 decreases capacitative Ca^{2+} entry and attenuates pulmonary artery smooth muscle cell proliferation. Am J Physiol Lung Cell Mol Physiol, 2002; 283(1): L144–55.

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15. Liu XR, Zhang MF, Yang N et al: Enhanced store-operated Ca(2)+ entry and TRPC channel expression in pulmonary arteries of monocrotaline-induced pulmonary hypertensive rats. Am J Physiol Cell Physiol, 2012; 302(1): C77–87

16. Smirnov SV, Robertson TP, Ward JP, Aaronson PI: Chronic hypoxia is associated with reduced delayed rectifier K+ current in rat pulmonary artery muscle cells. Am J Physiol, 1994; 266(1 Pt 2): H365–70

17. Owens GK: Regulation of differentiation of vascular smooth muscle cells. Physiol Rev, 1995; 75(3): 487–517

18. Ng LC, Kyle BD, Lennox AR et al: Cell culture alters Ca(2+) entry pathways activated by store-depletion or hypoxia in canine pulmonary arterial smooth muscle cells. Am J Physiol Cell Physiol, 2008; 294(1): C313–23

19. Danenberg HD, Welt FG, Walker M III et al: Systemic inflammation induced by lipopolysaccharide increases neointimal formation after balloon and stent injury in rabbits. Circulation, 2002; 105(24): 2917–22

20. Bon RS, Beech Dl: In pursuit of small molecule chemistry for calcium-permeable non-selective TRPC channels — mirage or pot of gold? Br J Pharmacol, 2013; 170(3): 459–74

21. Nakayama H, Wilkin BJ, Bodí I, Molkentin JD: Calcineurin-dependent cardiomyopathy is activated by TRPC in the adult mouse heart. FASEB J, 2006; 20(10): 1660–70

22. Daskoulidou N, Zeng B, Berglund LM et al: High glucose enhances store-operated calcium entry by upregulating ORAI/STIM via calcineurin-NFAT signalling. J Mol Med (Berl), 2015; 93(5): 511–21

23. Suzuki I, Bayna E, Li HL et al: Lipopolysaccharide activates calcineurin in ventricular myocytes. J Am Coll Cardiol, 2007; 49(4): 491–99