Sodium tanshinone IIA sulfonate inhibits hypoxia-induced enhancement of SOCE in pulmonary arterial smooth muscle cells via the PKG-PPAR-γ signaling axis

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PULMONARY ARTERIAL HYPERTENSION (PAH) is an uncommon yet deadly disease characterized by elevated pulmonary vascular resistance, leading to right ventricular failure. PAH is associated with progressive pulmonary vascular thickening and remodeling, which contribute to increase pulmonary vascular resistance. There are many physical and molecular etiologies of PAH, making it difficult to diagnose and resulting in inaccurate prognosis and treatment. Although there is no effective treatment for PAH, during the past decade, therapies, including anticoagulation, digoxin, oxygen, calcium channel blockers, L-arginine (nitric oxide precursor), nitroglycerin, diuretics, and others, have been identified and proven to be useful (2). As the understanding of the pathogenesis of PAH improves, a new class of drugs increasingly focuses on resolving vasodilation and contraction, reversing vascular proliferation, and reducing pulmonary vascular thickening and remodeling. These novel agents include prostacyclin, endothein receptor antagonists, and phosphodiesterase inhibitors (29). However, these medications are expensive and are accompanied by significant adverse reactions. Despite these new therapeutic options, the prognosis in patients with PAH remains poor, with a 1-yr survival rate of 91% (3) and a 3-yr survival rate of <77% (6, 16, 28). Therefore, there is an urgent need for novel and more cost-effective medications for the treatment of PAH.

Sodium tanshinone IIA sulfonate (STS) is a water-soluble salt solution of sulfonated tanshinone IIA, a monomer compound with a precisely known chemical structure that is known to be the active agent in a widely used traditional Chinese medicine known as Danshen. For decades, in China and other Asian countries, STS has been widely used as a medication for the treatment of cardiovascular disease, with rarely reported adverse reactions (14, 48). Recent studies demonstrate that STS has protective effects on chronic hypoxia-induced pulmonary hypertension (CHPH) and monocrotaline-induced pulmonary hypertension rat models by reducing the elevated mean pulmonary arterial pressure and right ventricular pressure,

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inhibiting pulmonary vascular remodeling, and blocking right ventricular hypertrophy (13, 35, 36). Moreover, studies have further demonstrated that STS executes these protective roles by targeting the intracellular calcium homeostasis in pulmonary arterial smooth muscle cells (PASMCs) (36). Our laboratory’s previous studies found that, among the three main calcium influx signaling pathways, store-operated calcium entry (SOCE), via store-operated calcium channel (SOCC), predominantly contributes to the elevation of intracellular calcium concentration ([Ca2+]i) seen in PASMCs associated with PAH. Furthermore, SOCC activation leads to triggered proliferation of PASMCs, pulmonary vasoconstriction, and pulmonary arterial remodeling under hypoxic conditions (39, 43). SOCC channels are primarily composed of transient receptor potential (TRPC) proteins (27, 35). In rodent pulmonary arteries (PAs) and PASMCs, hypoxia selectively upregulates TRPC1 and TRPC6 expression (24, 38, 40). Our laboratory’s previous results demonstrated that treatment with STS significantly inhibits hypoxia-increased TRPC1 and TRPC6 expression (24, 38, 40). Our laboratory’s previous studies found that, among the three main calcium influx signaling pathways, store-operated calcium entry (SOCE), via store-operated calcium channel (SOCC), predominantly contributes to the elevation of intracellular calcium concentration ([Ca2+]i) seen in PASMCs associated with PAH. Furthermore, SOCC activation leads to triggered proliferation of PASMCs, pulmonary vasoconstriction, and pulmonary arterial remodeling under hypoxic conditions (39, 43). SOCC channels are primarily composed of transient receptor potential (TRPC) proteins (27, 35). In rodent pulmonary arteries (PAs) and PASMCs, hypoxia selectively upregulates TRPC1 and TRPC6 expression (24, 38, 40). Our laboratory’s previous results demonstrated that treatment with STS significantly inhibits hypoxia-increased TRPC1 and TRPC6 expression, as well as hypoxia-enhanced SOCE and elevated [Ca2+]i, in both distal PAs and primary cultured PASMCs (36). These findings suggest that the beneficial effects of STS for the treatment of pulmonary hypertension are mediated by suppressing the SOCE and thereby decreasing intracellular calcium levels in diseased PASMCs.

Cyclic guanosine monophosphate (cGMP) is a ubiquitously expressed second messenger that activates the cGMP-dependent protein kinase (PKG). Chronic hypoxia exposure induces a significant reduction of the cGMP level in rat lungs, which in turn results in vasodilation of the pulmonary vasculature (15). KT5823, a specific inhibitor of PKG, elevates SOCE in PASMCs (5). Conversely, expression of cGMP-PKG represses the SOCE and thereby decreases intracellular calcium levels in diseased PASMCs.

Peroxisome proliferator-activated receptor-γ (PPAR-γ) belongs to a nuclear receptor superfamily, which was originally studied in the process of fatty acid storage and glucose metabolism (25). In the 1990s, the PPAR-γ agonist thiazolidinediones were approved for the treatment of diabetes. On ligand activation, PPAR-γ acts as a transcription factor, which translocates into the nucleus and binds with its cofactors, such as retinoid X receptors, to form heterodimers and regulate transcription of various genes (14). Recently, researchers have reported that the expression of PPAR-γ is significantly decreased in lungs from patients with PAH and animal models (1, 28). Moreover, accumulating evidence suggests that the PPAR-γ-specific ligand thiazolidinediones inhibits the pathogenesis of pulmonary hypertension in animal models (7, 10, 17). T0070907 (a specific inhibitor of PPAR-γ) elevates SOCE in PASMCs, whereas overexpressing PPAR-γ decreases SOCE in PASMCs (42).

Our laboratory previously demonstrated that sildenafil alleviates the pathogenesis of CHPH rat model by suppressing the expression of TRPC proteins and SOCE processes in PASMCs, and the cGMP-PKG-PPAR-γ axis involved in this protective effect of sildenafil (41). Therefore, in this study, we aim to determine the following: 1) if STS can rescue hypoxia-induced downregulation of PKG and PPAR-γ expression in distal PA and PASMCs; 2) if the PKG-PPAR-γ signaling axis can fit into the protective effect of STS on TRPC-SOCE-[Ca2+]i in PASMCs; and 3) whether STS exerts its anti-proliferative role in PASMCs through the PKG-PPAR-γ signaling axis. This study has the potential to further elucidate the molecular mechanisms by

Fig. 1. STS alleviated hypoxia-induced characteristic changes in chronic hypoxia PH rat model. A: representative traces of RVSP of each group of animals. Ppv, right ventricular pressure. B and C: bar graphs showing right ventricular systolic pressure (RVSP) and the ratio of weight of the right ventricle to the left ventricle plus interventricular septum [RV/LV + SI], respectively (n = 4 in each group). Norm, normoxia. Results have significant differences: *P < 0.05 compared with the *normoxia control group and & hypoxia control group. D: pulmonary vascular morphology in hematoxylin- and eosin-stained lung section. From left to right: normoxia, normoxia + STS, hypoxia, and hypoxia + STS groups. Arrows represent the pulmonary artery in each group.
which STS exerts its therapeutic effects on PAH. Furthermore, developing a better understanding of these mechanisms may lead to novel, more refined strategies for the treatment of PAH.

**MATERIALS AND METHODS**

Reagents and instruments. Specific pathogen-free male Sprague-Dawley rats (weight 200–250 g) were provided by Experimental Animal Center of Guangdong Province [license no. SCXK (of Can-

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**Fig. 2.** STS inhibited hypoxia-induced PKG and PPAR-γ downregulation in PASMCs and distal pulmonary arteries of rats. A and C: Western blot analysis of the effects of STS on PKG protein in rat distal pulmonary arteries and PASMCs, respectively. The top band is PKG, and the bottom band is β-tubulin in the normoxia control, normoxia + STS, hypoxia control, and hypoxia + STS groups. E and G: Western blot analysis of STS effects on PPAR-γ protein expression in rat pulmonary arteries and PASMCs, respectively, of which the top band is PPAR-γ and the bottom band is β-tubulin. The four groups are as defined in A and C. B, D, F, and H: STS effects on PKG protein expression in rat distal pulmonary arteries (n = 4; B) and PASMCs (n = 5; D) and STS effects on PPAR-γ protein expression in rat distal pulmonary arteries (n = 5; F) and PASMCs (n = 5; H). Values are means ± SE. Results have significant differences: *P < 0.05 vs. *normoxia control group and & hypoxia control group.
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STS INHIBITS HYPOXIA-INDUCED SOCE VIA PKG-PPAR-γ-TRPC AXIS

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4% O_2, 60h

57 kDa  PPARγ
55 kDa  β-tubulin

STS (μM)

100 kDa  TRPC1
55 kDa  β-tubulin

STS (μM)

100 kDa  TRPC6
55 kDa  β-tubulin

STS (μM)

100 kDa  TRPC1
55 kDa  β-tubulin

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55 kDa  β-tubulin

STS (μM)

100 kDa  TRPC6
55 kDa  β-tubulin

STS (μM)
enhanced chemiluminescence chemiluminescent liquid, and protein electrophoresis transfer systems from Bio-Rad; the RIPA lystate from Biyuan Biotechnology Institute; tanshinone IIA sulfonate from China Pharmaceutical and Biological Products, purity > 98%; tanshinone IIA sodium injection (STS) from China Shanghai Biochemical; PKG and PPAR-γ small interfering RNA (siRNA) oligo were synthesized by American GE Healthcare; GenSilencer siRNA transfection kit was purchased from American Genetantis. The remaining reagents were analytic grade products made in China; microplate reader TECAN type is Sunrise Remote/TonchScreen from Nanjing Huadong Electronics Group Medical Equipment (DG5033A type).

Animal models with chronic hypoxic pulmonary hypertension and grouping. All of the following procedures have been approved by the Medical Ethics Committee of Guangzhou Medical University. Male Sprague-Dawley rats (200–250 g) were randomly divided into four groups by the random number table: 1) normoxia control group, 2) normoxia + STS group, 3) hypoxia control group, and 4) hypoxia + STS group. Groups 1 and 2 were placed in normoxic condition and groups 3 and 4 in a hypoxic cabin with normal pressure, as previously reported, where the oxygen concentration was maintained at 10 ± 1%, in a sustained hypoxic condition for 21 days. Groups 2 and 4, starting from the first day of hypoxia, were, respectively, intraperitoneally injected with 30 mg/kg tanshinone IIA sulfonate; meanwhile, groups 1 and 3 received the same dose of saline.

Right ventricular systolic pressure, right ventricular hypertrophy, and lung histochemistry. Right ventricular systolic pressure (RVSP), the ratio of weight of the right ventricle to the left ventricle plus interventricular septum [RV/(LV + S)], and hematoxylin and eosin staining of lung tissue were measured, as previously described (36).

Primary culture of rat PASMCs. Rat PASMCs were cultured and identified by the common method of our study team (38, 39, 40). PASMCs were digested by collagenase and then cultured in low-sugar DMEM medium containing 10% fetal bovine serum. Furthermore, to ensure the cultured PASMCs retained a contractile phenotype, we performed experiments and set criteria for each culture. These experiments include the following: 1) positive immunofluorescence staining for smooth muscle cell markers smooth muscle α-actin and myosin heavy chain; and 2) an intracellular calcium increase of over 50 mM when chased with 60 mM KCl. When the fusion of cells was at 60–70%, the medium was replaced with the low-sugar DMEM medium containing 0.5% fetal bovine serum in which cells were cultured for 24 h to be homogenized. After the cells were grown to ~80%, they were randomly divided into four groups, two of which were treated with STS (12.5 μM).

STS group and STS-free group were randomly exposed to normoxic environment and hypoxic conditions (4% O2, 60 h). Our laboratory previously found that 60 h of prolonged hypoxic stress (4% O2) can effectively lead to elevated proliferation and migration of primary cultured distal PASMCs. This mimics similar hypoxic responses as the PASMCs isolated from the CPH rat, as the hypoxic elevation of [Ca2+]i, SOCE, and upregulation of TRPC expression in cultured PASMCs only occur at 60 h or later time points of hypoxic exposure (40). In this study, both incubators were set to 37°C, 5% CO2. The total protein of these cells was extracted by RIPA buffer. When cocultured with PKG inhibitor or PPAR-γ antagonist, PASMCs were pretreated for 1 h. According to our laboratory’s previous report (43), the effective concentrations for these reagents were 1 μM (RP8), 10 μM (T0070907), and 10 μM (GW1929), respectively.

Western blot. Western blot was performed as previously reported (16). After collecting the cells, RIPA buffer was used for cell lysis to extract total cellular protein, and the concentration of the protein was measured. Subsequently, 40 μg of protein were taken from each group to perform electrophoresis, 120 V for 10 min in 10% SDS-PAGE spacer gel, 150 V for 60 min in separation gel. Then we use electrotransformation to transfer protein to a polyvinylidene difluoride membrane. Five percent milk-Tris-buffered saline-Tween was used to block nonspecific antigen; primary antibody was incubated overnight, secondary antibody for 1 h, then enhanced chemiluminescence reagents for developing analysis.

siRNA transfection. When PASMCs are at 50–60% confluence, they are transfected with 1,000 ng PKG-siRNA or PPAR-γ-siRNA (On-Target plus SMARTpool, GE Healthcare) and negative control siRNA (NC-siRNA), by using GeneSilencer (Genlantis, San Diego, CA) as the transfection reagent for 6 h in serum-free smooth muscle basal medium. Serum was then added to a final concentration of 0.3%. PASMCs were exposed to siRNA for 60 h before subsequent analysis with Western blotting or intracellular calcium determination.

Intracellular Ca2+ determination. SOCE was measured in PASMCs using fura-2 dye and fluorescent microscopy, as previously described (39, 43). To obtain statistically valid results, the fluorescence intensity was determined in at least 20 cells for each sample.

Cell proliferation measurement. Cell proliferation experiments were performed as described previously (36, 42).

Statistical analysis. The gray values were analyzed by ImageJ software. The experimental data were shown as means ± SE; “n” represented the sample size, the number of animals that provided PA or PASMCs. SPSS13.0 statistical software was used for statistical analysis. Before running our statistical tests, we performed Shapiro-Wilk tests and determined the values to be >0.05, confirming normal distribution of the data. In this study, a T-test was used to compare the mean of two samples; groups were compared using univariate analysis of variance (one way-ANOVA) F-test; comparison between any two groups used the least significant difference method. P < 0.05 was considered statistically significant.

RESULTS

STS treatment prevents the pathogenesis of CPH in rat model. To determine whether STS treatment can decrease hemodynamic changes in CPH rat model, we established the CPH rat model and detected RVSP and RV/(LV + S). Data showed, compared with the control rats, RVSP was markedly elevated in hypoxia-induced PAH rats (Fig. 1B) (P < 0.05). However, this increase was significantly inhibited by STS prevention (30 mg·kg−1·day−1) (P < 0.05). In addition, there was no difference between the normoxia group and normoxia + STS group. Consistent with right ventricular pressure, intervention of STS also markedly lowered the ratio of RV/(LV + S) in hypoxia + STS group (0.415 ± 0.026), compared with hypoxia control group (0.55 ± 0.048) (Fig. 1C) (P < 0.05). Histological exami-

Fig. 3. PKG antagonists (RP-8) and PPAR-γ inhibitor (T0070907) reversed the STS-induced downregulation of TRPC1 and TRPC6 protein expressions under sustained hypoxic condition, and RP-8 weakened the effect of STS-upregulated PPAR-γ protein expression. A: Western blot analysis of PKG antagonist (RP-8) inhibition of STS-upregulated PPAR-γ protein expression under hypoxic condition in rat distal PASMCs, of which the top band is PPAR-γ and the bottom band is β-tubulin in the normoxia control, hypoxia control, hypoxia + STS, and hypoxia + STS + RP-8 groups, C, E, G, and I: Western blot analysis of PKG inhibitor (RP-8) and PPAR-γ antagonists (T0070907; G and I) reversing effects of STS downregulation of TRPC1 and TRPC6 protein expressions under sustained hypoxic condition in rat distal PASMCs. The top bands are TRPC1 (C and G) and TRPC6 (E and I); all of the bottom bands are β-tubulin. All pictures have four groups: normoxia control, hypoxia control, hypoxia + STS, and hypoxia + STS + T0070907. B: PKG antagonists (RP-8) inhibits STS-upregulated PPAR-γ protein expression under hypoxic condition in rat distal PASMCs. D, F, H, and J: mean intensity of TRPC1 (D and H) and TRPC6 (F and J). Values are means ± SE; n = 4. Results have significant differences: P < 0.05 vs. *normoxia control group, & hypoxia control group, and #hypoxia + STS group.
nation showed that the pulmonary vascular wall was thickened after 21-days of chronic hypoxia exposure, whereas STS treatment alleviated the hypoxia-induced pulmonary arterial wall thickening (Fig. 1D). STS treatment did not result in any significant hematomatological and histological changes in the normoxia group of rats (Fig. 1). This data indicated that STS exerts its beneficial effects on CHPH rat model.

**STS treatment rescues hypoxia-induced decrease in expression of PKG and PPAR-γ.** As shown in Fig. 2, A and B, chronic hypoxia (10% O2, 21 days) downregulated PKG protein expression to 61.69 ± 6.39% in rat distal PAs, compared with the control group (P < 0.01). However, the decline was significantly attenuated by STS intervention (30 mg·kg−1·day−1), which restored the PKG level back to 92.29 ± 6.96% (P < 0.01). We further investigated the effects of hypoxia and STS treatment on PKG expression in freshly isolated and cultured rat distal PASMCs. As illustrated in Fig. 2, C and D, prolonged hypoxia also (4% O2, 60 h) led to a significant decrease in PKG expression (60.41 ± 9.60%), which was then restored by STS (12.5 μM) treatment (P < 0.01). Similar effects of hypoxia occurred on the expression pattern of PPAR-γ. In Fig. 2, E and F, under chronic hypoxia (10%O2, 21 days), PPAR-γ protein expression in rat distal PA was downregulated to 57.73 ± 5.02% of the normoxia control group (P < 0.01). However, after STS intervention (30 mg·kg−1·day−1), PPAR-γ protein expression increased to 94.51 ± 4.47% (P < 0.01). In Fig. 2, G and H, PPAR-γ protein expression levels were markedly reduced to 41.78 ± 5.33% by prolonged hypoxic exposure (4% O2, 60 h) in PASMCs, compared with the normoxia control (P < 0.01). However, STS (12.5 μM) treatment almost completely attenuated the hypoxic decrease in expression of PPAR-γ (P < 0.01). Notably, STS did not affect the expression of either PKG or PPAR-γ in the normoxia groups throughout the experiment.

**Pharmacological inhibition of PKG or PPAR-γ rescues STS-mediated decrease in TRPC1 and TRPC6 expression in hypoxic PASMCs.** As STS can markedly affect the expression levels of PKG and PPAR-γ, we further investigated the potential involvement of PKG and PPAR-γ in STS-mediated protective signaling. PKG inhibitor RP-8 and PPAR-γ inhibitor T0070907 were used, respectively. Results in Fig. 3, A and B, show that 1-h pretreatment with PKG inhibitor RP-8 can suppress STS-restored PPAR-γ level. Moreover, as shown in Fig. 3, C–F, 1-h pretreatment of either RP-8 or PPAR-γ inhibitor T0070907 also significantly alleviated STS-mediated suppression on TRPC1 and TRPC6 expressions in hypoxic PASMCs, suggesting the involvement of PKG and PPAR-γ in the context of STS-mediated signaling.

The suppressive effects of STS on TRPC1 and TRPC6 expression in hypoxic PASMCs could be prevented by specific knockdown PKG or PPAR-γ. To further confirm the involvement of PKG and PPAR-γ in the STS-mediated signaling context, in addition to the use of pharmacological inhibitor, we knocked down expression by using specific PKG and PPAR-γ siRNAs. First, we evaluated the knockdown efficiency of PKG and PPAR-γ by Western blot. NC-siRNA was used as a nontargeting control. As shown in Fig. 4, A–D, specific siRNA transfection (1,000 ng, 60 h) led to a remarkable decrease of PKG and PPAR-γ protein expression to 33.58 ± 9.50 and 29.68 ± 4.02%, respectively, compared with the negative control group (P < 0.01). Knockdown of PKG attenuated STS-restored PPAR-γ level in hypoxic PASMCs (Fig. 4, E and F). On the other hand, specific knockdown of either PKG or PPAR-γ reversed STS-mediated suppression of hypoxia-induced TRPC expressions (Fig. 4, G–N), which confirmed the involvement of PKG and PPAR-γ in the context of STS-mediated signaling.

**PKG or PPAR-γ activity is required for STS-mediated decrease in basal calcium concentration and SOCE in hypoxic PASMCs.** Given the fact that STS can mediate the PKG-PPAR-γ signaling axis to regulate TRPC1 and TRPC6 protein in rat distal PASMCs, we then investigated whether STS-PKG-PPAR-γ can also target the basal [Ca2+]; and SOCE. As shown in Fig. 5, A–F, compared with the normoxia control group, hypoxic exposure significantly increased basal [Ca2+]; and SOCE (P < 0.01). The specific PKG inhibitor (RP-8, 1 μM) and PPAR-γ inhibitor (T0070907, 10 μM) markedly enhanced hypoxic upregulation of basal [Ca2+]; and SOCE; both RP-8 and T0070907 also significantly inhibited STS-mediated suppression of basal [Ca2+]; and SOCE in hypoxic PASMCs (P < 0.01). The suppressive effects of STS on basal calcium concentration and SOCE in hypoxic PASMCs can be reversed by specific knockdown of PKG or PPAR-γ. Similarly, we also used the specific PKG and PPAR-γ siRNA to confirm their roles during STS-mediated protection on intracellular calcium homeostasis in PASMCs exposed to hypoxia. Figure 6, A–C, showed that hypoxia markedly increased the basal [Ca2+]; and SOCE in PASMCs to 107.22 ± 7.19 and 261.43 ± 16.43 nM, compared with the NC-siRNA normoxia group, in which basal [Ca2+]; and SOCE were 75.70 ± 9.74 and 199.27 ± 15.98 nM, respectively (P < 0.01). Interestingly, basal [Ca2+]; and SOCE increased to 155.97 ± 8.28 nM and to 441.39 ± 15.47 nM in the PKG-siRNA hypoxia group, and STS remarkably decreased the basal [Ca2+]; and SOCE to 78.69 ± 9.16 nM and to 195.28 ± 18.12 nM in hypoxic PASMCs, compared with the
Fig. 5. The effect of STS-induced decline of basal \([\text{Ca}^{2+}]_i\) and SOCE in hypoxia rat PASMCs can be reversed by PKG or PPAR-γ inhibitor. A and C: changes of SOCE in rat distal PASMCs in the normoxia control, hypoxia control, hypoxia + STS, hypoxia + RP-8, and hypoxia + RP-8 + STS groups (n = 4). B: changes of basal \([\text{Ca}^{2+}]_i\) in the five groups. D and F: PPAR-γ antagonists (T0070907) reversing the STS-induced downregulation of SOCE under sustained hypoxic condition in rat distal PASMCs. There are five groups: normoxia control, hypoxia control, hypoxia + STS, hypoxia + T0070907, and hypoxia + T0070907 + STS (n = 5). E: basal \([\text{Ca}^{2+}]_i\) in the five groups (n = 5). Values are means ± SE. Results are statistically significant: \(P < 0.05\) vs. *normoxia control group, & hypoxia control group, and #hypoxia + STS group.
Fig. 6. Knockdown PKG or PPAR-γ inhibited STS-induced intracellular Ca^{2+} homeostasis in rat PASMCs under hypoxia. A and C: changes of SOCE in rat distal PASMCs from five groups: normoxia NC-siRNA control, hypoxia NC-siRNA control, hypoxia NC-siRNA + STS, hypoxia PKG-siRNA, and hypoxia PKG-siRNA + STS (n = 5). B: basal [Ca^{2+}] in the five groups (n = 4). D and F: changes of SOCE in rat distal PASMCs from five groups: normoxia NC-siRNA control, hypoxia NC-siRNA control, hypoxia NC-siRNA + STS, hypoxia PPAR-γ-siRNA, and hypoxia PPAR-γ-siRNA + STS (n = 5). E: changes of basal [Ca^{2+}] in the five groups (n = 4). Values are means ± SE. Results are statistically significant: *P < 0.05 vs. *normoxia control group, & hypoxia control group, and #hypoxia STS group.
NC-siRNA hypoxia group ($P < 0.01$). However, this effect of STS can be suppressed by the combination treatment of STS and PKG-siRNA (150.86 ± 11.69 and 415.37 ± 27.12 nM, respectively). Similar to the effect of PKG-siRNA, in Fig. 6, $D–F$, basal [Ca$^{2+}$], and SOCE in PPAR-γ-siRNA hypoxia group was markedly increased to 152.91 ± 14.78 and 452.02 ± 15.47 nM, compared with the NC-siRNA hypoxia group, in which basal [Ca$^{2+}$], and SOCE were 110.13 ± 9.85 and 265.72 ± 18.43 nM, respectively ($P < 0.01$). Moreover, compared with the combined use of STS and NC-siRNA hypoxia group ($P < 0.01$). Furthermore, decreasing PPAR-γ activity with T0070907 treatment remarkably restored STS-downregulated proliferation in hypoxic PASMCs (Fig. 7B, $P < 0.05$). Compared with hypoxia + STS group, in which PASMCs proliferation rate was 116.41 ± 9.97%, combination treatment with GW1929 and STS significantly enhanced hypoxic PASMCs proliferation (67.78 ± 3.53%; $P < 0.05$).

PPAR-γ agonist promotes the protective role of STS on basal [Ca$^{2+}$], and SOCE in hypoxic PASMCs. As shown in Fig. 8 and similarly with our laboratory’s previous experiments, hypoxia significantly increased basal [Ca$^{2+}$], and SOCE in PASMCs to 128.82 ± 7.50 and 255.34 ± 10.38 nM, compared with normoxia control group, in which basal [Ca$^{2+}$], and SOCE were 82.53 ± 5.57 and 196.07 ± 7.29 nM, respec-

![Graph A](http://ajpcell.physiology.org/)

**Fig. 7.** Antagonizing PKG or PPAR-γ reverse the inhibitory effect of STS on proliferation in hypoxic PASMCs, whereas agonizing PPAR-γ enhances the effect. **A:** cell proliferation in rat distal PASMCs from normoxia control, normoxia + STS, normoxia + RP-8, normoxia + STS + RP-8, hypoxia control, hypoxia + STS, hypoxia + RP-8, and hypoxia + STS + RP-8 groups. **B:** cell proliferation in eight groups: normoxia control, normoxia + STS, normoxia + T0070907, normoxia + STS + T0070907, hypoxia control, hypoxia + STS, hypoxia + T0070907, and hypoxia + STS + T0070907. **C:** cell proliferation of PASMCs in normoxia control, normoxia + STS, normoxia + GW1929, normoxia + STS + GW1929, hypoxia control, hypoxia + STS, hypoxia + GW1929, and hypoxia + STS + GW1929 groups. Values are means ± SE; $n = 4$. Results are statistically significant: $P < 0.05$ vs. *normoxia control group, & hypoxia control group, and #hypoxia + STS group.

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tively \((P < 0.05)\). However, treatment with STS or GW1929 markedly lowered hypoxia-induced upregulation of basal \([\text{Ca}^{2+}]\), and SOCE in PASMCs \((P < 0.05)\). In addition, combination treatment with STS and GW1929 significantly strengthened STS-induce decrease of basal \([\text{Ca}^{2+}]\), and SOCE in hypoxic PASMCs \((P < 0.05)\).

DISCUSSION

Danshen (salvia) and its active ingredients have been widely used in the treatment of cardiovascular diseases in China and other Asian countries for many years. This treatment is known for its high efficacy and rarely reported side effects. Among the several active ingredients, tanshinone IIA is one of the most abundant and effective compounds. STS is a water-soluble form of tanshinone IIA, and STS has been clinically used for decades in the treatment of numerous cardiovascular diseases, such as hypertension, atherosclerosis, and others (45). It is reported that STS effectively prevented the development of hypertension by inhibiting the proliferation of basilar arterial smooth muscle cells (49). Moreover, by activating AMP-activated protein kinase, STS is reported to restore high-glucose induced proliferation of vascular smooth muscle cells (44). Another study also demonstrated that STS suppressed the proliferation of vascular smooth muscle cells by inhibiting the ERK1/2 signaling pathway (34). Recently, in a small population-based pilot study, our group demonstrated for the first time that STS has potential therapeutic effects on the treatment of pulmonary hypertension in concert with other on-market medications such as sildenafil (37). Moreover, our animal study further confirmed that STS significantly attenuates the development of experimental pulmonary hypertension in both CHPH and monocrotaline-induced pulmonary hypertension rat by targeting intracellular calcium homeostasis, especially the SOCE process in PASMCs (36). In line with our laboratory’s previous study (36), in this report we demonstrated that STS exerts its beneficial roles in the hypoxia-induced PAH rat model by reducing hypoxia-
induced increase of mean right ventricular pressure, RVSP, and RV/(LV + S) and reversing hypoxia-induced pulmonary vascular remodeling.

\([\text{Ca}^{2+}]\) acts as a major factor in facilitating the proliferation and contraction of PASMCs, which together result in excessive thickening and remodeling of distal PAs and contribute to the development and progression of pulmonary hypertension (19). Our laboratory’s previous study demonstrates that \([\text{Ca}^{2+}]\) influx through SOCC (termed SOCE) largely accounts for the enhanced \([\text{Ca}^{2+}]\), in hypoxic PASMCs, which is thought to be a major contribution to the excessive proliferation and contraction of cells in hypoxia (38, 40). Furthermore, we find that STS-targeted SOCE-\([\text{Ca}^{2+}]\) signaling resulted in the reduction of pulmonary arterial pressure and vascular remodeling (36).

Previous studies indicate that PKG plays an important role on the proliferation of PASMCs. Li et al. (22) report that PKG suppresses the proliferation of PASMCs through inhibition of RhoA and ERK1/2 signaling. Chattergoon et al. demonstrate that the PKG inhibitor, RP-8, restores the proliferation of PASMCs induced by calcitonin gene-related peptide (4). Our laboratory’s previous data indicate that PKG is involved in the protective context of sildenafil on SOCE-\([\text{Ca}^{2+}]\) signaling (23, 41).

PPAR-\(\gamma\) is a member of the nuclear receptor family, the important transcription factors that regulate fat synthesis and glucose metabolism (11, 12, 20). Many studies of different cell lines find that PPAR-\(\gamma\) has an anti-proliferative effect on cells through different signaling pathways (9, 26, 32, 33). The PPAR-\(\gamma\) agonist rosiglitazone can inhibit PASMCs proliferation and hypoxia-induced pulmonary vascular remodeling and reduce mean PA pressure in hypoxic pulmonary hypertension rat models (7, 21). Additionally, our laboratory recently verified that PPAR-\(\gamma\) inhibits CPHH pathogenesis by inhibiting hypoxia inducible factor 1 (HIF-1) signal and thus results in reducing TRPC and SOCE in PASMCs (49).

In this study, our results suggest that hypoxia downregulates PKG and PPAR-\(\gamma\) protein levels, which can be restored by STS in both PAs and PASMCs. Using inhibitors/agonist and siRNA knockdown against PKG and PPAR-\(\gamma\), we demonstrate that both PKG and PPAR-\(\gamma\) are involved in STS-mediated protective signaling context on TRPC, SOCE, and cell proliferation in hypoxic PASMCs. Our laboratory’s previous studies demonstrate that \([\text{Ca}^{2+}]\) imbalance is primarily caused by SOCE and accompanied TRPC protein upregulation in pulmonary hypertension (38, 40). STS can reduce hypoxia-induced increase of TRPC1 and TRPC6 expression in pulmonary vascular smooth muscle layer and PASMCs, thereby reducing the cellular basal calcium concentration and SOCE, leading to the reduction of right ventricular pressure and inhibition of vascular proliferation and remodeling in hypoxic pulmonary hypertension rat models (13, 36). Our findings reveal, at least in part, the mechanism for which STS attenuates proliferation of PASMCs and PA remodeling during CPHH pathogenesis.

In our laboratory’s previous studies, we show that PKG and PPAR-\(\gamma\) are involved in the regulation of TRPC expression in PASMCs and alterations to calcium concentration through SOCC (41, 49). Recently, our laboratory also confirmed that PPAR-\(\gamma\) modulates TRPC expression and SOCE in PASMCs by inhibiting caveolin-1 (46). Moreover, PPAR-\(\gamma\) and HIF-1\(\alpha\) regulate calcium homeostasis in PASMCs by sharing mutual inhibitory mechanisms (47). Our results demonstrate that STS treatment attenuates hypoxia-upregulated SOCE through the PKG-PPAR-\(\gamma\)-TRPC pathway. Indeed, it is likely that HIF-1\(\alpha\) and caveolin-1 may be involved in this signaling; however, more experiments are needed in future studies to further explore the relationship between PPAR-\(\gamma\), HIF-1\(\alpha\), and caveolin-1.

In this study, we aimed to determine the molecular mechanism of STS on the regulation of SOCE. Therefore, by using both pharmacological inhibitors and knockdown strategies of PKG and PPAR-\(\gamma\), we systematically investigated the involvement of PKG and PPAR-\(\gamma\) in the STS-mediated protective signaling axis on intracellular calcium regulation and proliferation in hypoxic PASMCs. Notably, siRNA knockdown against neither PKG nor PPAR-\(\gamma\) (which mimics the hypoxic downregulation on PKG and PPAR-\(\gamma\)) could alter TRPC, basal \([\text{Ca}^{2+}]\), and SOCE in normoxic PASMCs, suggesting the possibility that PKG and PPAR-\(\gamma\) expression levels can affect TRPC and SOCE only under hypoxic conditions. The different downstream effects of STS between normoxic and hypoxic rats or PASMCs can, to some extents, support this hypothesis.

Nevertheless, our study has some limitations, such as characteristics of primary culture PASMCs may not be exactly the same as that of the smooth muscle cells in normoxic arteries, and it still needs to be determined whether PKG and PPAR-\(\gamma\) are the feedback regulators of STS treatment. However, in a continuation of previous work, this study has now provided mechanistic evidence to support the efficiency and feasibility of STS, which has satisfied clinical efficiency, rarely reported side effect, and relatively lower cost (compared with classic pulmonary hypertension medication), as a potential treatment for pulmonary hypertension.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


