Sildenafil Inhibits Hypoxia-Induced Transient Receptor Potential Canonical Protein Expression in Pulmonary Arterial Smooth Muscle via cGMP-PKG-PPARγ Axis

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Transient receptor potential canonical (TRPC) proteins play important roles in chronically hypoxic pulmonary hypertension (CHPH). Previous results indicated that sildenafil inhibited TRPC1 and TRPC6 expression in rat distal pulmonary arteries (PAs). However, the underlying mechanisms remain unknown. We undertook this study to investigate the downstream signaling of sildenafil's regulation on TRPC1 and TRPC6 expression in pulmonary arterial smooth muscle cells (PASMCs). Hypoxia-exposed rats (10% O_2 for 21 d) and rat distal PASMCs (4% O_2 for 60 h) were taken as models to mimic CHPH. Real-time PCR, Western blotting, and Fura-2–based fluorescent microscopy were performed for mRNA, protein, and Ca^{2+} measurements, respectively. The cellular cyclic guanosine monophosphate (cGMP) analogue 8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate sodium salt (CPT-cGMP) (100 μM) inhibited TRPC1 and TRPC6 expression, store-operated Ca^{2+} entry (SOCE), and the proliferation and migration of PASMCs exposed to prolonged hypoxia. The inhibition of CPT-cGMP on TRPC1 and TRPC6 expression in PASMCs was relieved by either the inhibition or knockdown of cGMP-dependent protein kinase (PKG) and peroxisome proliferator–activated receptor γ (PPARγ) expression. Under hypoxic conditions, CPT-cGMP increased PPARγ expression. This increase was abolished by the PKG antagonists Rp8 or KT5823. PPARγ agonist GW1929 significantly decreased TRPC1 and TRPC6 expression in PASMCs. Moreover, hypoxia exposure decreased, whereas sildenafil treatment increased, PKG and PPARγ expression in PASMCs *ex vivo*, and in rat distal PAs *in vivo*. The suppressive effects of sildenafil on TRPC1 and TRPC6 in rat distal PAs and on the hemodynamic parameters of CHPH were inhibited by treatment with the PPARγ antagonist T0070907. We conclude that sildenafil inhibits TRPC1 and TRPC6 expression in PASMCs via cGMP-PKG-PPARγ–dependent signaling during CHPH.

CLINICAL RELEVANCE

Accumulating evidence indicates that the up-regulation of canonical transient receptor potential (TRPC) proteins is responsible for chronic hypoxia–induced increases of Ca^{2+} influx and elevations of intracellular Ca^{2+} concentrations in pulmonary arterial smooth muscle cells (PASMCs), and thus may contribute to the pathogenesis of chronically hypoxic pulmonary hypertension (CHPH). However, little is known about the regulation of TRPCs, especially in the context of hypoxic induction. Sildenafil, a potent and selective Type V phosphodiesterase inhibitor, has recently been accepted in the treatment of various classes of pulmonary hypertension. However, its detailed mechanisms of action are not fully understood. Previous studies demonstrated that sildenafil attenuates CHPH in association with reduced store-operated Ca^{2+} entry (SOCE) and normalized TRPC1 and TRPC6 expression in rat distal PASMCs. This study was designed to elucidate the signaling pathway through which sildenafil attenuates the hypoxia-induced enhancement of SOCE and the up-regulations of TRPC1 and TRPC6 by hypoxia. The results indicate that sildenafil inhibits TRPC1 and TRPC6 expression and SOCE in PASMCs via a cGMP-PKG-peroxisome proliferator–activated receptor γ–dependent mechanism during the development of CHPH. This study not only advances our understanding about the regulation of TRPCs, but also the mechanism underlying the beneficial effects of sildenafil in treating pulmonary hypertension.

Keywords: sildenafil; PKG; PPARγ; TRPC; PASMCs

Pulmonary hypertension (PH) involves a group of diseases characterized by increased contraction and structural remodeling in the pulmonary vasculature. Among various mechanisms uncovered so far, persistent alveolar hypoxia is thought to comprise an important trigger for PH. Animals such as rats and mice develop chronically hypoxic pulmonary hypertension (CHPH) upon exposure to chronic hypoxia (CH).

Ca^{2+} constitutes a key signal for both the contraction and growth of vascular smooth muscle cells (1–3). We and others previously found that elevations of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) play a critical role in the pathogenic process of CHPH (2, 4–6). In pulmonary arterial smooth muscle

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cells, store-operated Ca^{2+} channel (SOCC)–mediated Ca^{2+} influx, or so-called store-operated Ca^{2+} entry (SOCE), comprised the primary pathway responsible for the $[Ca^{2+}]_i$ increase induced by both acute and chronic hypoxia in pulmonary arterial smooth muscle cells (PASMCs) (5, 7). SOCC is thought to be composed of canonical transient receptor potential (TRPC) protein family members. Out of the seven members of the TRPC family identified so far, TRPC1, TRPC4, and TRPC6 are predominantly expressed in rat distal PASMCs (8, 9). In distal pulmonary arteries (PAs) from rats that developed CHPH, TRPC1 and TRPC6 but not TRPC4 were selectively up-regulated (5). A knockdown study with short, interfering RNA (siRNA) treatment revealed that both TRPC1 and TRPC6 contributed to the hypoxia-induced increases of SOCE and basal $[Ca^{2+}]_i$ in PASMCs (10).

Sildenafil is currently used as a major agent for the treatment of PH by specifically targeting to Type V phosphodiesterase. We recently found that inhibitions of TRPC1 and TRPC6 expression and functional decreases of SOCE and basal $[Ca^{2+}]_i$ in PASMCs are likely involved in the therapeutic effects of sildenafil on CHPH (10). However, the underlying mechanisms remain unknown. Primarily, sildenafil is thought to exert the relaxation and growth inhibition of vascular smooth muscle cells by raising the cellular concentration of cyclic guanosine monophosphate (cGMP), which subsequently causes the activation of PKG. Application of cGMP analogue led to similar consequences, such as the relaxation of precontracted pulmonary arteries (11) and the inhibition of PASMC proliferation (12). Intrapulmonary arteries from CHPH rats exhibit reduced cGMP concentrations because of the increased activity of specific cGMP phosphodiesterases (PDEs) such as PDE5 (13, 14). Moreover, evidence from whole-cell patch clamp electrophysiology demonstrated that a cGMP-PKG–dependent signaling pathway negatively modulates SOCC activity in distal PASMCs (15).

Peroxisome proliferator–activated receptor γ (PPARγ), a classic transcriptional factor in regulating fatty-acid storage and glucose metabolism, is widely expressed in various cell types, including PASMCs. In lung tissue from patients with pulmonary arterial hypertension (PAH), in animals with CHPH, or in PASMCs exposed to prolonged hypoxia, the protein concentrations of PPARγ were reduced (16–18). The reduction of PPARγ expression or activity may contribute to the pathogenic mechanism of PAH, because the administration of synthetic PPARγ agonists such as pioglitazone, troglitazone, and rosiglitazone could attenuate monocrotaline-induced and hypoxia-induced PH in rats and mice (18–21). Conversely, the deletion of PPARγ in smooth muscle caused PH in a mouse model (22, 23). The relationship between cGMP-PKG signaling and PPARγ and their modulatory effects on TRPC expression in PASMCs during CHPH have not been elucidated.

This study investigated the involvement of cGMP-PKG and PPARγ in the down-regulatory effects of sildenafil on TRPC1 and TRPC6 expression in PASMCs exposed to prolonged hypoxia, and in distal PAs from CHPH rats. We found that sildenafil inhibited hypoxic increments of TRPC1 and TRPC6 expression in PASMCs. These inhibitions required the activation of the cGMP-PKG-PPARγ–dependent signaling pathway. These results contribute to our understanding of the pathogenic mechanisms of CHPH, and to interpreting the therapeutic effects of sildenafil.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (weighing 250–300 g) were obtained from Harlan (Frederick, MD). All animal experiment procedures were approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Exposure of Rats to Chronic Hypoxia, and Treatments with Sildenafil and T0070907

A rat model of CHPH was established and treated with sildenafil (50 $mg \cdot kg^{-1} \cdot d^{-1}$) and T0070907 (2 $mg \cdot kg^{-1} \cdot d^{-1}$), as we previously described (5, 10). Detailed protocols are provided in the online supplement.

Hemodynamic Measurements and Lung Histochemistry

Right ventricular systolic pressure and right ventricular hypertrophy were measured using the same method as we previously described (10). Intrapulmonary vessels were visualized by hematoxylin-and-eosin staining on formalin-fixed and paraffin-embedded lung cross sections of 5-μm thickness. More detailed protocols are included in the online supplement.

Isolation and Culture of PASMCs

Rat distal pulmonary arteries (PAs, > 4th generation) were isolated from anesthetized male Wistar rats and denuded from the adventitia and endothelium. PASMCs were enzymatically dissociated and cultured as we previously described (5, 9, 10). More detailed culture and treatment protocols are also provided in the online supplement.

siRNA Transfection

The siRNAs specific for PKG (siPKG) or PPARγ (siPPARγ) were designed according to Genebank accession numbers NM_001105731.3 for PKG and NM_013124.3 for PPARγ mRNA, and were purchased as ON-TARGETplus SMARTpool products from Thermo Fisher Scientific, Inc. (Lafayette, CO). The detailed siRNA treatment protocols are described in the online supplement.

Measurements of SOCE

PASMCs were loaded with 7.5 μM Fura-2 (Molecular Probes, Inc., Eugene, OR), perfused with free Krebs Ringer bicarbonate solution in the presence of 10 μM cyclopiazonic acid and 5 μM nifedipine, and then subjected to SOCE assessment by Mn^{2+} quenching, as we described elsewhere (8). SOCE was evaluated from the rate at which Fura-2 fluorescence was quenched by Mn^{2+}. Details are provided in the online supplement.

Real-Time Quantitative PCR

Total RNA was extracted from PASMCs, using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). Reverse transcription was performed using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time quantitative PCR was performed using the QuantiTect SYBR Green PCR Master Mix (Qiagen) in an iCyclerIQ real-time PCR detection system (Bio-Rad). The detailed protocols are described in the online supplement.

Western Blot Analysis

Distal PAs or PASMCs were homogenized in TPER lysis buffer (Pierce, Rockford, IL) containing 5% protease inhibitor cocktail (Sigma, St. Louis, MO), 1 mM EDTA, and 200 μM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride. Western blotting was performed according to a method we have previously described (10). Please see the online supplement for a more detailed description.

Cell Proliferation, Migration, and Cytotoxicity Assessments

As we previously described (24), PASMC proliferation was assessed using an Amersham Cell Proliferation Biotrak ELISA kit (GE Healthcare, Buckinghamshire, UK). Cell migration was assessed on an 8-μm polycarbonate membrane of Transwell Permeable Support (24 mm; Corning, Inc., Corning, NY) (1×10^5 cells/well). The cytotoxicity of various drug treatments on PASMCs was analyzed using a CytoTox-Glo Cytotoxicity Assay kit (Promega, Madison, WI). More detailed protocols are provided in the online supplement.

Statistical Analysis

Data are expressed as means ± SEMs, and n expresses the number of experiments performed equaling the numbers of animals providing PAs or cells. Statistical comparisons were performed with the Student

t test for analyzing two-group data, whereas one-way ANOVA was used to test differences among multiple independent groups. $P < 0.05$ was considered significant.

RESULTS

CPT-cGMP Inhibited Hypoxic Increments of TRPC1 and TRPC6 Expression and SOCE in PASMCs

Previously, we demonstrated that sildenafil inhibited the hypoxic induction of TRPC1 and TRPC6 expression and SOCE in PASMCs (10). As a specific PDE5 inhibitor, sildenafil functions to accumulate cellular cGMP by blocking the degradation of cGMP. In this study, we used cell-permeable, cGMP-derivative 8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate sodium salt (CPT-cGMP) to examine whether it resembles the effects of sildenafil on TRPC and SOCE levels in PASMCs exposed to prolonged hypoxia. Similar to our previous observations with sildenafil, incubation with CPT-cGMP at 10 μM, 100 μM and 1,000 μM dose-dependently inhibited both TRPC1 (Figure 1A) and TRPC6 (Figure 1B) mRNA expression in PASMCs under prolonged hypoxic conditions (4% O_2 for 60 hours). The maximal inhibitions of CPT-cGMP on concentrations of TRPC1 (Figure 1A) and TRPC6 (Figure 1B) mRNA were achieved at 1,000 μM. The expression levels of neither TRPC1 (Figure 1A) nor TRPC6 (Figure 1B) mRNA were significantly different between treatments with 100 μM and 1,000 μM. Therefore, the dose of

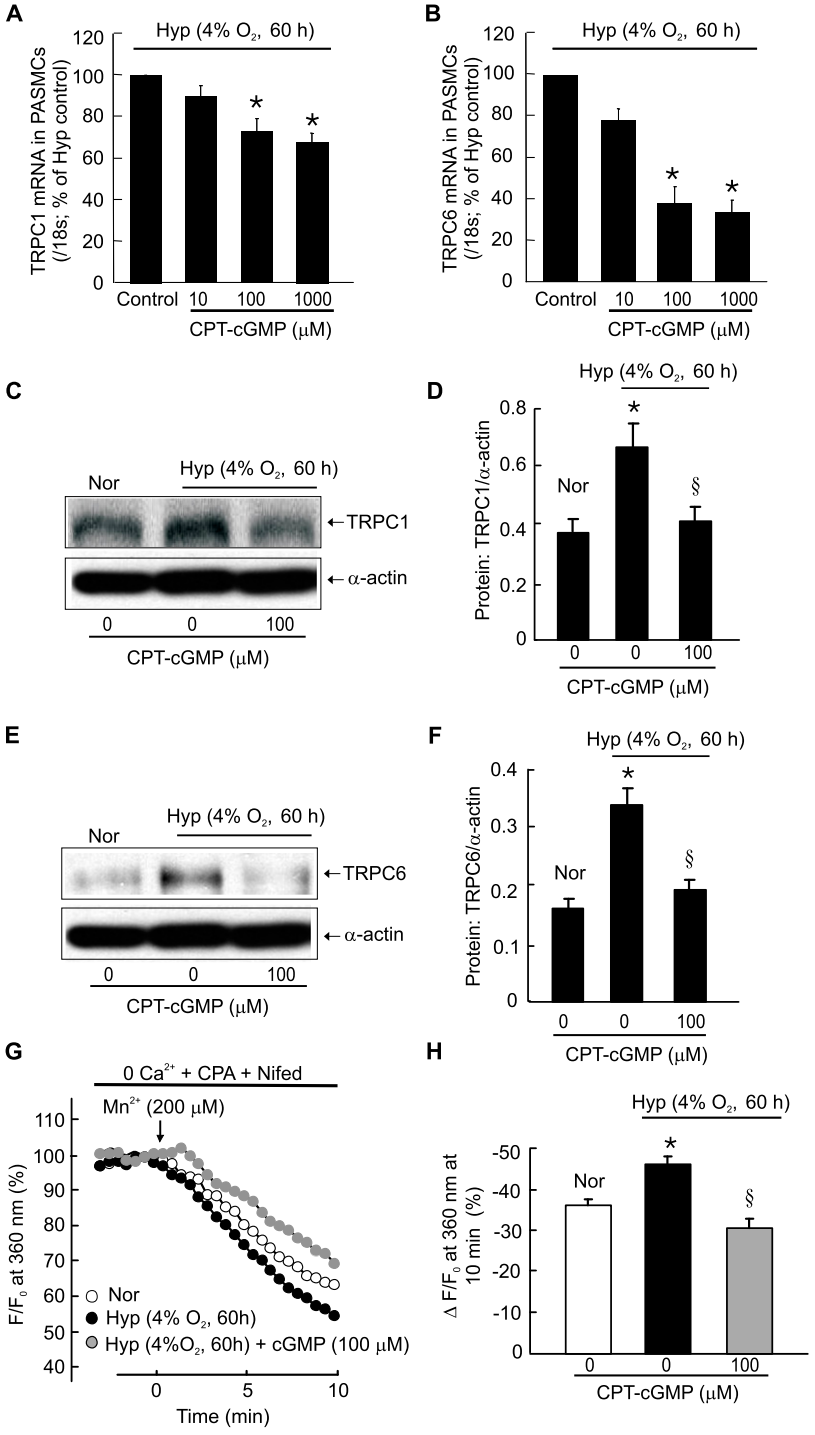

Figure 1. Effects of 8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate sodium salt (CPT-cGMP) on canonical transient receptor potential (TRPC)–1 and TRPC6 protein expression and store-operated Ca^{2+} entry (SOCE) in distal pulmonary arterial smooth muscle cells (PASMCs) under prolonged hypoxic conditions. (*A* and *B*) Concentrations of TRPC1 (*A*) and TRPC6 (*B*) mRNA relative to 18S were determined by real-time quantitative PCR in PASMCs treated with vehicle control or CPT-cGMP at 10, 100, or 1,000 μM during prolonged hypoxia (Hyp; 4% O_2 for 60 hours). *Bar values* represent the means ± SEMs ($n = 3$ in each group). *$P <$ 0.05, versus respective Hyp control. (*C* and *E*) Representative Western blots of TRPC1, TRPC6, and α-actin in PASMCs exposed to normoxia (Nor) or hypoxia (Hyp; 4% O_2) for 60 hours, with or without CPT-cGMP (100 μM) treatment. (*D* and *F*) TRPC1 (*D*) and TRPC6 (*F*) protein expressions were determined by Western blotting and normalized to α-actin in PASMCs treated with Nor, Hyp control, or Hyp + CPT-cGMP. *Bar values* represent the means ± SEMs ($n = 3$ in each group). *$P < 0.05$, versus respective Nor control. §$P <$ 0.05, versus respective Hyp control. (*G* and *H*) Effects of CPT-cGMP treatment (100 μM) on SOCE assessed by Mn^{2+} quenching. (*G*) Time course of Fura-2 fluorescence excited at 360 nm, before and after the addition of 200 μM Mn^{2+} in Ca^{2+}-free Krebs Ringer bicarbonate (KRB) solution (0 Ca^{2+}) perfusates containing 10 μM cyclopiazonic acid (CPA) and 5 μM nifedipine (Nifed) in rat distal PASMCs treated with Nor ($n = 4$ experiments with 97 cells), Hyp ($n = 4$ experiments with 74 cells), or Hyp plus CPT-cGMP ($n = 4$ experiments with 92 cells). The fluorescence as a function of time (F) was normalized to the fluorescence value measured immediately before addition of Mn2+ (F0). (*H*) Percent changes in Mn^{2+} quenching. ΔF/F0 indicated the relevant percentage changes of fluorescence normalized to basal value. Data are expressed as percent decreases in fluorescence at 10 minutes after time 0. *Bar values* represent the means ± SEMs. *$P < 0.01$, versus Nor control. §$P < 0.01$, versus Hyp control.

100 μM CPT-cGMP was chosen for subsequent experiments. Using this dose, treatment with CPT-cGMP normalized TRPC1 (Figures 1C and 1D) and TRPC6 (Figures 1E and 1F) protein expression, as well as SOCE (Figures 1G and 1H), in rat distal PASMCs exposed to prolonged hypoxia. Notably, compared with normoxic control samples, hypoxic exposure increased TRPC1 protein expression by approximately 64% (Figures 1C and 1D), and TRPC6 protein expression by approximately 85% (Figures 1E and 1F). The SOCE measured by Mn^{2+} quenching was increased by approximately 24% via hypoxia (Figures 1G and 1H). Treatment with CPT-cGMP (100 μM) essentially blocked these hypoxic increments of TRPC1 (Figures 1C and 1D) and TRPC6 (Figures 1E and 1F) protein expression, as well as SOCE (Figures 1G and 1H), in PASMCs.

Inhibition of PKG Prevented the Suppressive Effects of CPT-cGMP on TRPC1 and TRPC6 Expression in PASMCs

To identify whether the inhibition of CPT-cGMP on hypoxia-induced increases of TRPC1 and TRPC6 expression was attributable to a PKG-dependent pathway, the PKG antagonists Rp8 (1 μM) and KT5823 (0.5 μM) were included 30 minutes before CPT-cGMP treatment on PASMCs. Doses of 1 μM for Rp8 and 0.5 μM for KT5823 were selected according to serial dosage tests because they caused maximal inhibitions in the CPT-cGMP–induced activation of vasodilator-stimulated phosphoprotein (Figure E1A and E1B), a known substrate protein for PKG (25). Under hypoxic conditions, both Rp8 and KT5823 prevented the inhibitory effects of CPT-cGMP on TRPC1 (Figures 2A and 2B) and TRPC6 (Figures 2C and 2D) protein expression. In conjunction with these preventive effects on TRPC1 and TRPC6 expression, the inhibition of SOCE by CPT-cGMP treatment was also attenuated (Figures 2E and 2F) in PASMCs pretreated with Rp8 and KT5823. These results indicate that the activation of PKG is required for executing the negative regulations of CPT-cGMP on the TRPC1, TRPC6, and SOCE increases induced by hypoxia in PASMCs.

PPARγ Activation Was Involved in cGMP-PKG Signaling–Mediated Inhibition on Hypoxic Increases of TRPC1 and TRPC6 Expression in PASMCs

Both PKG and PPARγ play important inhibitory roles in PASMC hypertrophy and the development of PAH. Therefore, we examined whether PPARγ was regulated by cGMP and PKG, mediating their down-regulatory effects on TRPC1 and TRPC6 expression under prolonged hypoxic conditions. As seen in Figures 3A and 3B, CPT-cGMP (100 μM) treatment significantly increased PPARγ protein expression in PASMCs. This increase was blocked by pretreatment with PKG inhibitors Rp8 (1 μM) and KT5823 (0.5 μM), indicating the induction of PPARγ by CPT-cGMP was

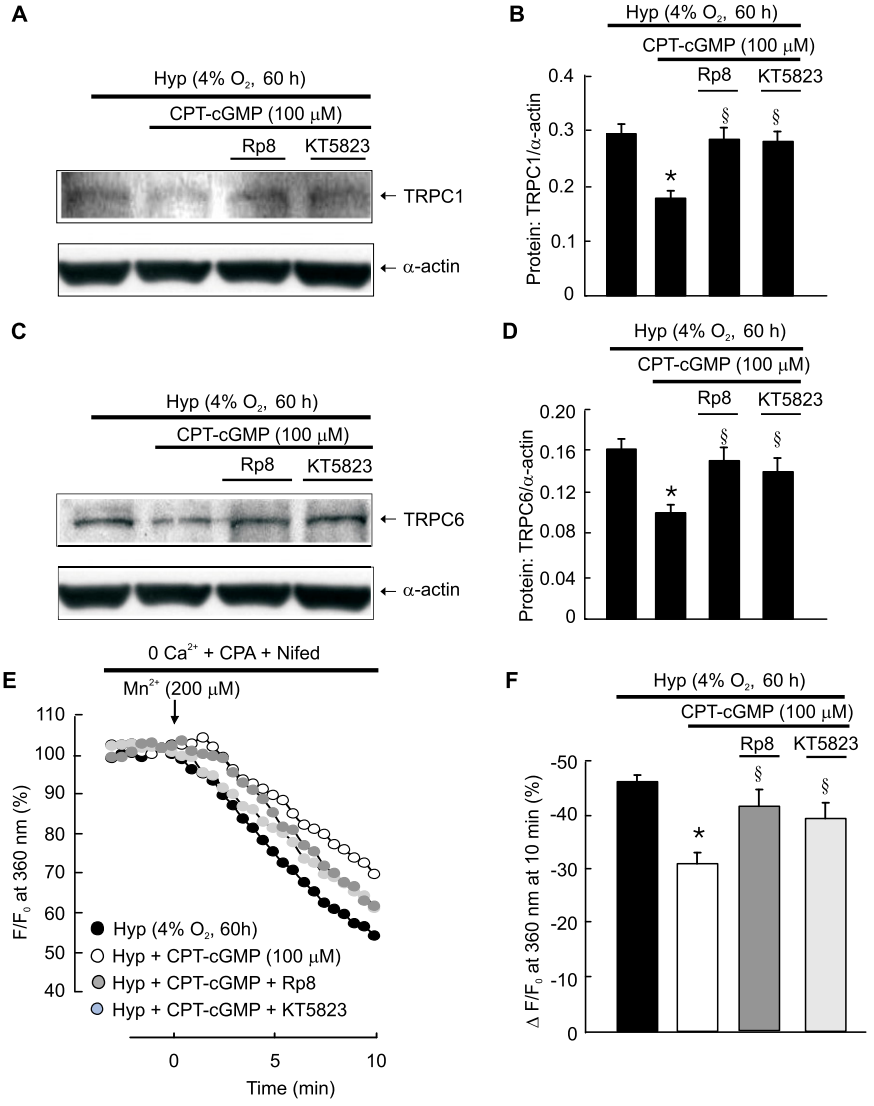

Figure 2. Inhibition of PKG blocked the down-regulatory effects of CPT-cGMP on TRPC1 and TRPC6 protein expression in PASMCs exposed to prolonged hypoxia. (*A* and *C*) Representative Western blots for TRPC1 (*A*), TRPC6 (*C*), and α-actin (*A* and *C*) in distal PASMCs exposed to hypoxia (Hyp; 4% O_2) and treated with or without CPT-cGMP (100 μM) and PKG inhibitors Rp8 (1 μM) or KT5823 (0.5 μM) for 60 hours. (*B* and *D*) Expression of TRPC1 (*B*) and TRPC6 (*D*) proteins relative to α-actin (*B* and *D*) in distal PASMCs treated with or without CPT-cGMP (100 μM) and PKG inhibitors Rp8 (1 μM) or KT5823 (0.5 μM), as determined by Western blotting. *Bar values* represent the means ± SEMs ($n = 4$ in each group). *$P < 0.05$, versus Hyp control. §$P < 0.05$, versus Hyp plus CPT-cGMP. (*E* and *F*) Treatment with PKG inhibitors attenuated the down-regulatory effects of CPT-cGMP on SOCE, as assessed by Mn^{2+} quenching. (*E*) Time course of Fura-2 fluorescence excited at 360 nm before and after the addition of 200 μM Mn^{2+} in Ca^{2+}-free KRBS (0 Ca^{2+}) perfusates containing 10 μM CPA and 5 μM nifedipine in distal PASMCs treated with or without CPT-cGMP (100 μM) and PKG inhibitors Rp8 (1 μM) or KT5823 (0.5 μM) for 60 hours under hypoxic conditions. (*F*) Percent changes in Mn^{2+} quenching in rat distal PASMCs treated with Hyp alone ($n = 4$ experiments with 74 cells), Hyp + CPT-cGMP ($n = 4$ experiments with 92 cells), Hyp + CPT-cGMP + Rp8 ($n = 4$ experiments with 90 cells) or Hyp + CPT-cGMP + KT5823 ($n = 4$ experiments with 103 cells). Data are expressed as the percent decrease in fluorescence at 10 minutes after time 0. *Bar values* represent the means ± SEMs. *$P < 0.05$, versus Hyp control. §$P < 0.05$, versus Hyp + CPT-cGMP.

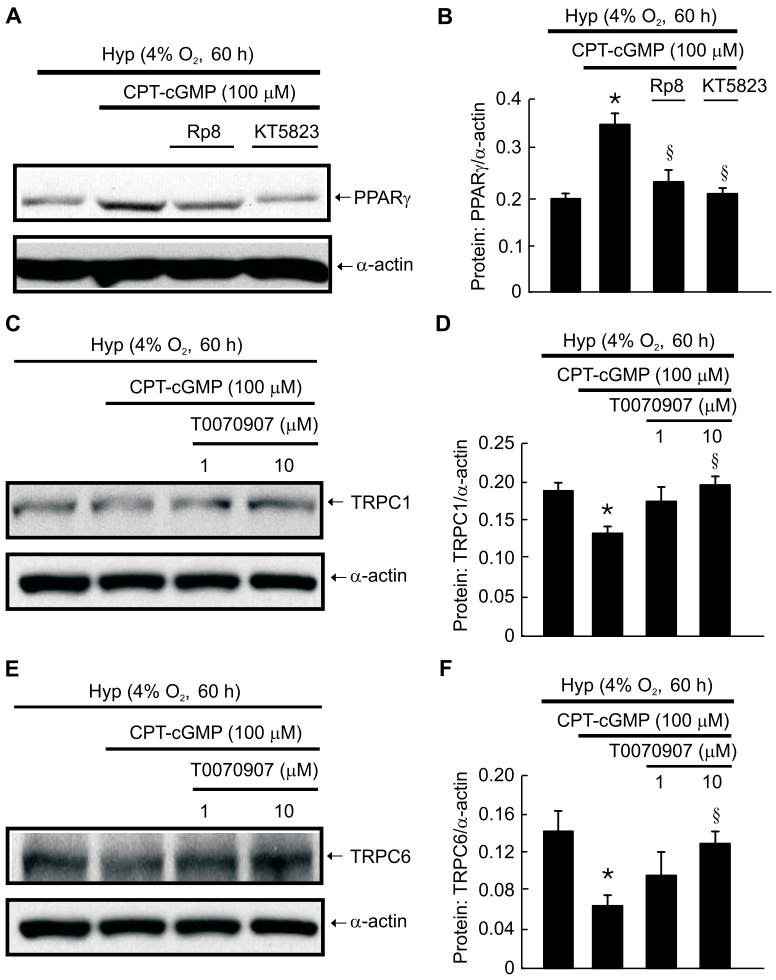

Figure 3. Inhibition of PKG decreased the CPT-cGMP–induced activation of peroxisome proliferator–activated receptor γ (PPARγ), and the blockade of PPARγ attenuated the down-regulation of CPT-cGMP on TRPC1 and TRPC6 protein expression in rat distal PASMCs under prolonged hypoxic conditions. (*A*, *C*, and *E*) Representative Western blots of PPARγ (*A*), TRPC1 (*C*), TRPC6 (*E*), and α-actin (*A*, *C*, and *E*) protein in distal PASMCs exposed to hypoxia (Hyp; 4% O_2) and treated with or without CPT-cGMP (100 μM), PKG antagonists Rp8 (0.5 μM) and KT5823 (1.0 μM), and PPARγ antagonist T0070907 (1 μM and 10 μM) for 60 hours. (*B*, *D*, and *F*) Mean intensity for PPARγ (*B*), TRPC1 (*D*), and TRPC6 (*F*) blots relative to α-actin (*B*, *D*, and *F*). *Bar values* represent the means ± SEMs ($n = 3$ in each group). *$P < 0.05$, versus respective Hyp control. §$P < 0.05$, versus Hyp plus CPT-cGMP.

PKG-dependent. Treatment with PPARγ antagonist T0070907 at 1 μM and 10 μM dose-dependently inhibited the down-regulations of CPT-cGMP (100 μM) in TRPC1 (Figures 3C and 3D) and TRPC6 (Figures 3E and 3F) protein expression, with greater inhibition obtained at 10 μM of T0070907. In contrast, the activation of PPARγ by PPARγ agonist GW1929 (10 μM) inhibited TRPC1

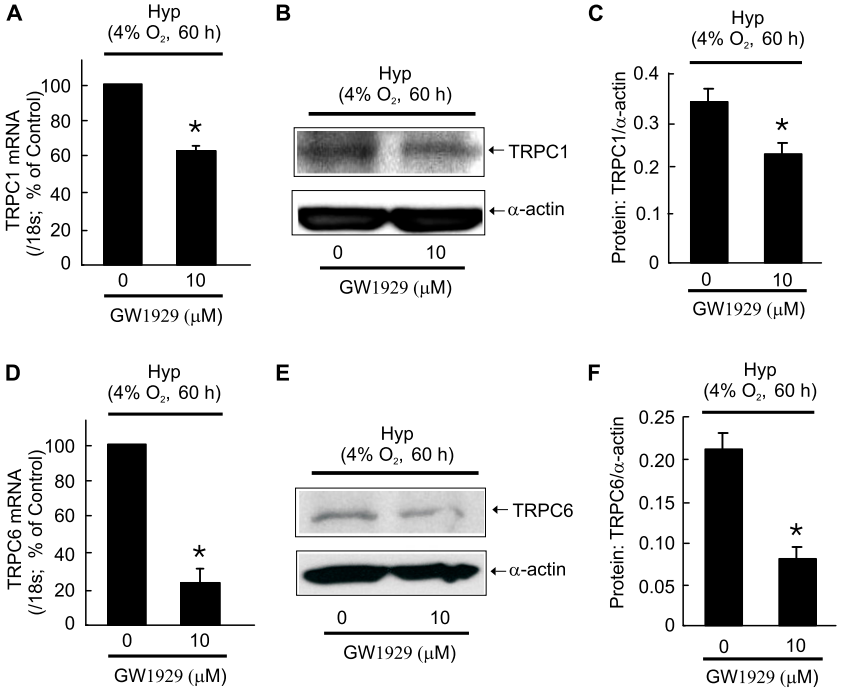

Figure 4. Activation of PPARγ inhibits hypoxic up-regulations of TRPC1 and TRPC6 mRNA and protein expression in PASMCs. (*A* and *D*) Effects of PPARγ agonist GW1929 (10 μM) on TRPC1 (*A*) and TRPC6 (*D*) mRNA expression in rat distal PASMCs exposed to hypoxic control (Hyp; 4% O_2) or Hyp plus GW1929 for 60 hours, as determined by real-time quantitative PCR. *Bar values* represent the means ± SEMs ($n = 3$ in each group). *$P < 0.05$, versus Hyp control. (*B* and *E*) Representative Western blots of TRPC1 (*B*), TRPC6 (*E*), and α-actin protein (*B* and *E*) in distal PASMCs exposed to Hyp or Hyp plus GW1929 for 60 hours. *C* and *F* show mean intensities for TRPC1 (*C*) and TRPC6 (*F*) blots, relative to α-actin. *Bar values* represent the means ± SEMs ($n = 3$ in each group). *$P < 0.05$, versus respective Hyp control.

and TRPC6 mRNA (Figures 4A and 4D) and protein (Figures 4B and 4C for TRPC1, and Figures 4E and 4F for TRPC6) expression in PASMCs under prolonged hypoxic conditions. These results indicate that PPARγ acts downstream of cGMP-PKG and mediates its down-regulation of TRPC1 and TRPC6 expression during hypoxic exposure. Doses of 10 μM for GW1929 and 10 μM for T0070907 were selected for these and subsequent experiments in this study, because they caused maximal changes of the protein concentrations of the unphosphorylated active form of PPARγ in PASMCs in tests with serial doses of 10 μM and 20 μM for GW1929 (Figures E2A and E2B), and 0.1 μM, 1 μM, and 10 μM for T0070907 (Figures E2C and E2D).

Knockdown of PKG and PPARγ Prevented CPT-cGMP–Induced Suppression of TRPC1 and TRPC6 Expression in PASMCs under Prolonged Hypoxic Conditions

Treatment with siRNA specific for PKG and PPARγ resulted in reduction of PKG protein expression (Figures E3A and E3B) by approximately 71% and PPARγ (Figures E3C and E3D) by approximately 74% in PASMCs. Similar to the effects of PKG antagonists Rp8 and KT5823, and PPARγ antagonist T0070907, knockdown of either PKG or PPARγ eliminated the CPT-cGMP (100 μM)–induced suppression of TRPC1 (Figures E4A and E4C with siPKG; Figures E4E and E4F with siPPARγ) and TRPC6 (Figures E4A and E4D with siPKG, and Figures E4E and E4G with siPPARγ) protein expression in PASMCs exposed to prolonged hypoxia (4% O_2 for 60 hours). Moreover, the knockdown of PKG blocked CPT-cGMP–induced increases in PPARγ protein expression in PASMCs under prolonged hypoxia (Figures E4A and E4B). These results confirm that the inhibitory effects of cGMP on hypoxic increases of TRPC1 and TRPC6 expression are PKG-dependent and PPARγ-dependent.

CPT-cGMP Inhibited Hypoxia-Induced Increases of PASMC Proliferation and Migration in Dependence on PKG and PPARγ

To examine the functional influences of CPT-cGMP-PKG-PPAR-TRPC signaling, we further investigated the effects of CPT-cGMP and the antagonism of PKG and PPARγ in PASMC proliferation and migration. As shown in Figure E5, exposure to prolonged hypoxia (4% O_2, 60 hours) led to a 41% increase in the proliferation (Figure S5A) and a 55% increase in the migration (Figure E5B) of PASMCs. Treatment with CPT-cGMP (100 μM) inhibited these increases (Figures E5A and E5B). The inhibitory effects of CPT-cGMP on hypoxic increases of PASMC proliferation (Figure E5A) and migration (Figure E5B) were blocked by pretreatment with PKG antagonists Rp8 (1 μM), KT5823 (0.5 μM), or PPARγ antagonist T0070907 (10 μM), indicating a dependence on both PKG and PPARγ. Treatment with PPARγ agonist GW1929 (10 μM) inhibited the hypoxic increments of PASMC proliferation (Figure E5A) and migration (Figure E5B), suggesting that the down-regulation of PPARγ was involved. Via these pharmacological treatments with CPT-cGMP, Rp8, KT5823, T0070907, and GW1929, the viability of PASMCs was not affected (Figure E5C), suggesting that the effects of these drugs on cell proliferation and migration were not likely attributable to a change in cell viability.

Sildenafil Up-Regulated PKG and PPARγ Expression in PASMCs and in Rat Distal PAs under Hypoxic Conditions

Exposure to prolonged hypoxia (4% O_2 for 60 hours) reduced both PKG (Figures 5A and 5B) and PPARγ (Figures 5C and 5D) protein expression in PASMCs. Under prolonged hypoxic conditions, treatment with sildenafil (300 nM) led to remarkable increases in both PKG (Figures 5E and 5F) and PPARγ (Figures 5G and 5H) protein expression in PASMCs.

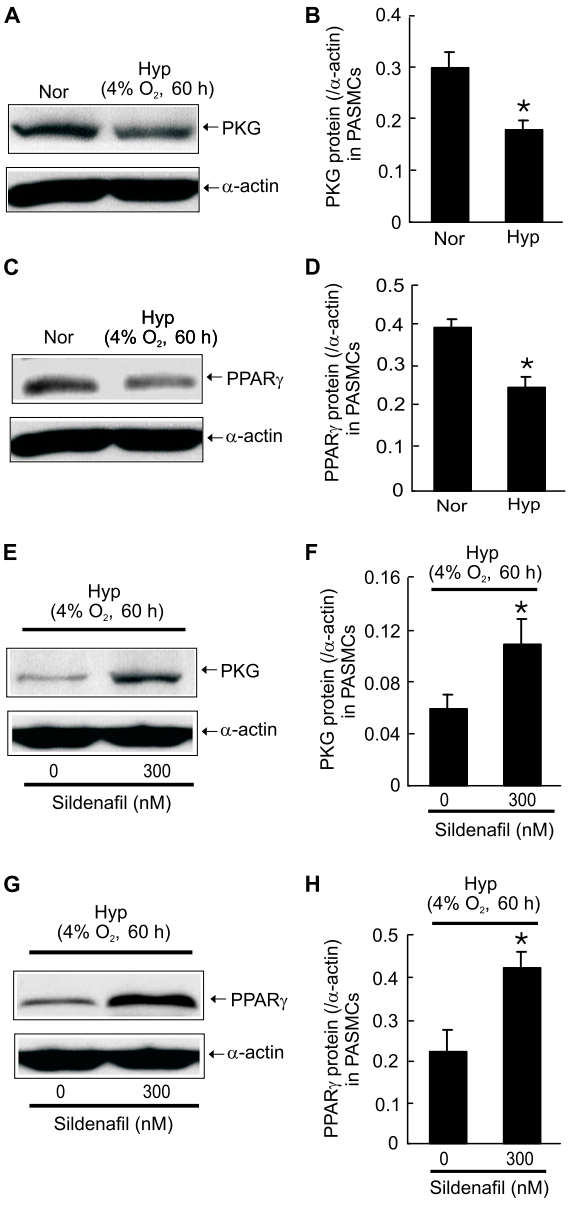

Figure 5. Effects of prolonged hypoxia and sildenafil treatments on PKG and PPARγ protein expression in rat distal PASMCs. (*A* and *C*) Representative Western blots for PKG (*A*), PPARγ (*C*), and α-actin (*A* and *C*) proteins in PASMCs exposed to normoxia (Nor) or hypoxia (Hyp; 4% O_2) for 60 hours. *B* and *D* show mean intensities for PKG (*B*) and PPARγ (*D*) blots relative to α-actin (*B* and *D*) in PASMCs exposed to Nor or Hyp. *Bar values* represent the means ± SEMs ($n = 3$ in each group). *$P < 0.05$, versus respective Nor control. (*E* and *G*) Representative Western blots of PKG (*E*), PPARγ (*G*), and α-actin (*E* and *G*) in PASMCs exposed to prolonged hypoxia (Hyp; 4% O_2 for 60 h) or Hyp + sildenafil (300 nM). (*F* and *H*) Mean intensities for PKG (*F*) and PPARγ (*H*) blots relative to α-actin in PASMCs exposed to Hyp or Hyp + sildenafil. *Bar values* represent the means ± SEMs ($n = 3$ in each group). *$P < 0.05$, versus respective Hyp control.

Similar to what we reported previously (10), rats exposed to CH (10% O_2 for 21 d) developed CHPH, as indicated by elevated right ventricular systolic pressure (RVSP) (Figure E6A), an index of right ventricular hypertrophy (weight ratio of right

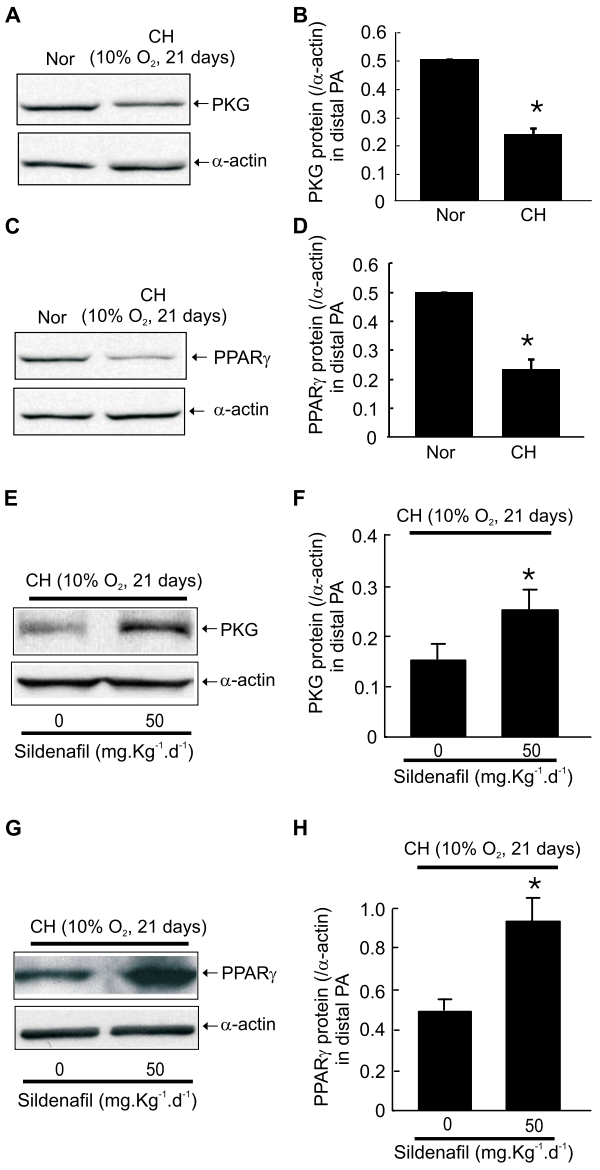

Figure 6. Effects of chronic hypoxia and sildenafil treatments on PKG and PPARγ expression in rat distal pulmonary arteries (PAs). (*A* and *C*) Representative Western blots of PKG (*A*), PPARγ (*C*), and α-actin (*A* and *C*) proteins in distal PAs from rats exposed to normoxia (Nor) or chronic hypoxia (CH; 10% O_2 for 21 d). (*B* and *D*) Mean intensities for PKG (*B*) and PPARγ (*D*) blots relative to α-actin (*B* and *D*) in distal PAs from rats exposed to Nor or CH. *Bar values* represent the means ± SEMs ($n = 4$ in each group). *$P < 0.05$, versus respective Nor control. (*E* and *G*) Representative Western blots for PKG (*E*), PPARγ (*G*), and α-actin (*E* and *G*) proteins in distal PAs from rats exposed to CH or CH + sildenafil (50 mg · kg^{-1} · d^{-1}). (*F* and *H*) Expression of PKG (*F*) and PPARγ (*H*) relative to α-actin, as determined by Western blotting in distal PAs from rats exposed to CH or CH + sildenafil. *Bar values* represent the means ± SEMs ($n = 4$ in each group). *$P < 0.05$, versus respective CH control.

ventricle to left ventricle plus septum [RV/(LV+S)] in Figure E6B), and by increased medial wall thicknesses of the small pulmonary vessels (Figure E6D), compared with those in normoxic control rats (Figures E6A–E6C). Treatment with sildenafil (50 mg · kg^{-1} · d^{-1}) dramatically attenuated these hypoxic increases of RVSP (Figure E6A), RV/LV + S (Figure E6B), and small pulmonary vessel remodeling (Figure E6E) in rats exposed to CH. Consistent with these observations in PASMCs *in vitro*, CHPH rats displayed reduced protein concentrations of PKG (Figures 6A and 6B) and PPARγ (Figures 6C and 6D) in distal PAs, compared with those in normoxic control rats. On the other hand, treatment with sildenafil resulted in increased PKG (Figures 6E and 6F) and PPARγ (Figures 6G and 6H) protein expression in distal PAs from rats exposed to CH.

Treatment with PPARγ Antagonist T0070907 Attenuated the Inhibitory Effects of Sildenafil on TRPC1 and TRPC6 Expression in Distal PAs from Chronically Hypoxic Rats

Consistent with what we observed previously (10), rats exposed to CH exhibited increased expressions of TRPC1 (Figures 7A and 7B) and TRPC6 (Figures 7C and 7D) protein. In conjunction with these inhibitory effects on the hemodynamic parameters of CHPH and the up-regulatory effects on PKG and PPARγ protein expression, treatment with sildenafil (50 mg · kg^{-1} · d^{-1}) eliminated the hypoxic increases of TRPC1 (Figures 7A and 7B) and TRPC6 (Figures 7C and 7D) protein expression. Combined treatment of PPARγ antagonist T0070907 (2 mg · kg^{-1} · d^{-1}) with sildenafil attenuated the inhibitory effects of sildenafil on CH-induced increases of TRPC1 (Figures 7A and 7B) and TRPC6 (Figures 7C and 7D) protein expression, as well as the increases in RVSP (Figure E6A), RV/LV + S (Figure E6B), and small pulmonary vessel remodeling (Figure E6F).

DISCUSSION

This study provides evidence that sildenafil inhibits the hypoxic up-regulations of TRPC1 and TRPC6 expression in a cGMP-PKG-PPARγ–dependent signaling pathway.

First of all, we found that CPT-cGMP significantly inhibited the hypoxia-induced increases of TRPC1 and TRPC6 expression. These inhibitions could be blocked by either PKG and PPARγ inhibition or the knockdown of PKG and PPARγ protein expression, indicating the participation of PKG and PPARγ in the down-regulations of cGMP on TRPC1 and TRPC6. Next, we examined the link between PKG and PPARγ. We demonstrated that CPT-cGMP also enhanced PPARγ expression, and this effect was attenuated by PKG inhibitors or the knockdown of PKG protein expression, suggesting a regulatory role of PKG in PPARγ. Moreover, we observed decreased PKG and PPARγ expressions in distal PAs from CHPH rats and in PASMCs exposed to prolonged hypoxia, and such decreases were attenuated by sildenafil treatment. Finally, treatment with the PPARγ antagonist T0070907 prevented the inhibitory effects of sildenafil in hypoxic up-regulations of TRPC1 and TRPC6, and inhibited the beneficial effects of sildenafil on CHPH.

Accumulating evidence indicates that the activation or overexpression of TRPC family members in PASMCs is crucial in the pathogenesis of PH (5, 6, 26–28). TRPC6 was up-regulated in PAs from patients with idiopathic pulmonary arterial hypertension (26). A functional single-nucleotide polymorphism (the −254(C→G) SNP) in the TRPC6 gene promoter, which confers increased NF-κB–mediated promoter activity leading to TRPC6 overexpression, was found to be associated with idiopathic pulmonary arterial hypertension in patients (29). In PAs from CHPH rats, we and others previously demonstrated that TRPC1 and TRPC6 were selectively up-regulated (5, 6), whereas TRPC1 and TRPC4 expression was increased in the monocrotaline-induced rat model of PH (27). The more direct effects of overexpressed TRPC in promoting PH are thought to enhance pulmonary vasoconstriction through increasing SOCE (30). Blockades of SOCC or inhibitions of TRPC expression or channel activity inhibited the stimuli-elicited vasoconstriction of intralobar pulmonary artery rings (31–34). Moreover, both

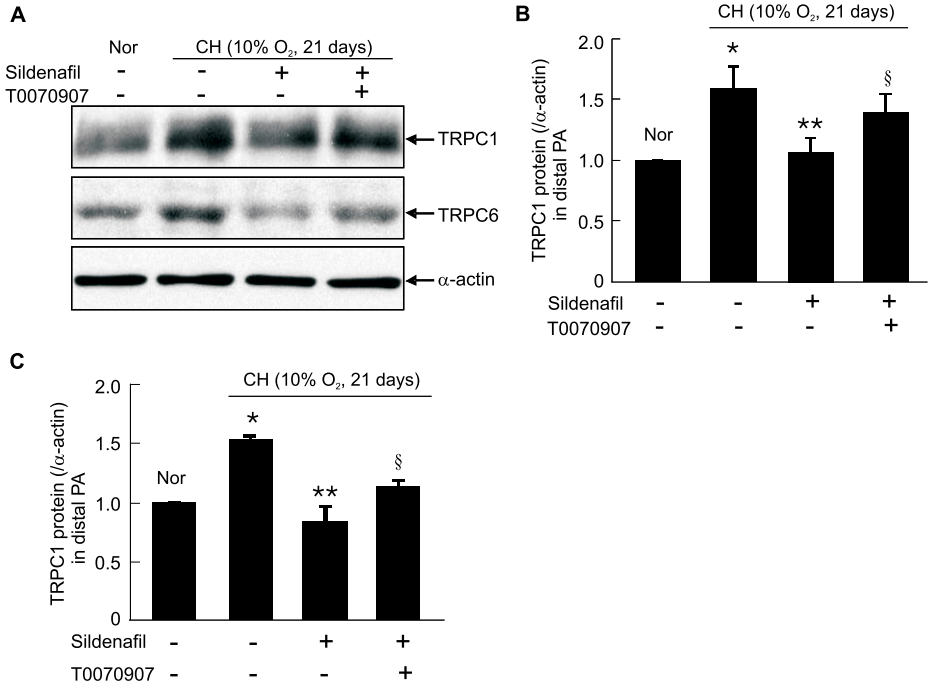

Figure 7. Effects of PPARγ antagonist T0070907 and sildenafil on TRPC1 and TRPC6 expression in distal PAs from rats exposed to chronic hypoxia. (*A*) Representative Western blots of TRPC1, TRPC6, and α-actin in distal PAs from rats exposed to normoxia (Nor), chronic hypoxia (CH; 10% O_2 for 21 d), CH + sildenafil (50 mg · kg^{-1} · d^{-1}), or CH + sildenafil (50 mg · kg^{-1} · d^{-1}) + T0070907 (2 mg · kg^{-1} · d^{-1}). (*B* and *C*) Protein expression of TRPC1 (*B*) and TRPC6 (*C*) was determined by Western blotting in rats exposed to Nor, CH, CH + sildenafil, or CH + sildenafil + T0070907. *Bar values* represent the means ± SEMs ($n = 4$ in each group). *$P < 0.05$, versus respective Nor control. **$P < 0.05$, versus respective CH control. §$P < 0.05$, versus respective CH + sildenafil.

TRPC1 and TRPC6 have been shown to mediate SOCE and to facilitate PASMC proliferation and migration (30, 35, 36). In association with the down-regulations of TRPC1 and TRPC6 by CPT-cGMP, we found that the SOCE, proliferation, and migration of PASMCs were also decreased by CPT-cGMP treatment.

The second messenger cGMP regulates pleiotropic cellular functions in both health and disease (37). Increases of cGMP led to PKG activation, $[Ca^{2+}]_i$ reduction, weakened sensitivity to Ca^{2+}, and consequently decreased smooth muscle tone (38, 39). Previous studies indicate that CH decreased cellular cGMP concentrations and reduced responses to NO donors in lung and distal pulmonary arteries, attributable at least in part to the increased activity of PDE5, which mediates the hydrolysis of cGMP (14, 40). In addition, hypoxia also repressed PKG expression in PASMCs, which correlated with the phenotypic change of cell from a contractile and differentiated to a synthetic and dedifferentiated state (41). Consistent with these observations from the literature, we demonstrated that hypoxia significantly decreased PKG expression in rat distal PAs *in vivo* and in PASMCs *ex vivo*, whereas sildenafil treatment inhibited such decreases.

In contrast to the growing evidence regarding the role of TRPC in the pathogenesis of PH, information on understanding the regulatory mechanisms of TRPC is limited. Among such information gathered so far, c-Jun/signal transducer and activator of transcription–3 was shown to be involved in the platelet-derived growth factor– induced up-regulation of TRPC6 (35). NF-κB was reported to mediate TNF-α–induced TRPC1 expression (42). Both extracellular-regulated kinase (ERK) and NF-κB were shown to be involved in the angiotensin II–induced up-regulation of TRPC6 in podocytes (43). Finally, ERK1/2 activation was found to be required for increased TRPC1 expression in breast cancer cells (44). Although the activation of PKG has been known to inhibit SOCE in PASMCs by inhibiting TRPC6 activity (15, 45, 46), little is known regarding whether or how cGMP-PKG signaling regulates TRPC expression. Because PKG was down-regulated by CH while being up-regulated by sildenafil treatment, we predicted that sildenafil may play its inhibitory role on TRPC expression through a cGMP-PKG–dependent signaling pathway. Indeed, we found that a 60-hour treatment of CPT-cGMP mimicked the effects of sildenafil in inhibiting TRPC1 and TRPC6 expression under hypoxic conditions. These inhibitions were attenuated by PKG inhibitors (KT5823 and Rp8) and by the knockdown of PKG protein. These results indicate that the down-regulation of sildenafil on TRPC1 and TRPC6 expression depends on cGMP-PKG.

PPARγ belongs to the nuclear receptor family, which functions as a transcription factor to regulate adipogenesis and glucose metabolism (47–49). It exerts antiproliferative effects on different cell types via various signaling pathways (50–53). Recently, PPARγ was shown to modulate PH via multiple mechanisms (54–56), and its activation was demonstrated to prevent or reverse PH in hypoxia or monocrotaline-induced animal models (18, 22, 23, 57). Sildenafil was found to increase PPARγ expression in adipocytes, contributing to the mechanism of adipogenesis (58). Based on such evidence, we sought to identify whether sildenafil and cGMP-PKG exerted their effects on down-regulating TRPCs by influencing PPARγ expression. First of all, we found that PPARγ agonist GW1929 could significantly decrease hypoxia-induced mRNA and protein up-regulations of TRPC1 and TRPC6, whereas PPARγ antagonist T0070907 markedly attenuated the down-regulatory effects of CPT-cGMP on TRPC1 and TRPC6 protein expression in PASMCs. These results proved that PPARγ could negatively regulate TRPC1 and TRPC6 expression, suggesting that PPARγ may exert its inhibitory role in PH by affecting the expression of TRPC in PASMCs. Next, we determined the relationship between PKG and PPARγ, and found that PKG inhibitors attenuated CPT-cGMP–induced PPARγ up-regulation. Finally, we found that the protein concentrations of both PKG and PPARγ were reduced by hypoxic exposure in PAs and PASMCs. Sildenafil treatments inhibited these reductions. These data indicate that PPARγ is regulated by PKG, and that it acts downstream from PKG in mediating the inhibitory effects of sildenafil or cGMP in hypoxia-induced TRPC1 and TRPC6 overexpression. The detailed mechanism of how PPARγ regulates TRPC1 and TRPC6 expression requires further investigation.

In conclusion (and as illustrated in Figure E7), this is the first report, to our knowledge, demonstrating that down-regulations of

PKG and PPARγ are involved in hypoxia-induced up-regulations of TRPC1 and TRPC6 expression, which are associated with an enhancement of SOCE and the proliferation and migration of PASMCs. Sildenafil reversed these hypoxic responses through a cGMG-PKG-PPARγ–dependent pathway. This mechanism contributes, at least in part, to elucidating the beneficial effects of sildenafil in the treatment of PH.

Author disclosures are available with the text of this article at www.atsjournals.org.

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