

A Functional Single-Nucleotide Polymorphism in the *TRPC6* Gene Promoter Associated With Idiopathic Pulmonary Arterial Hypertension

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Background—Excessive proliferation of pulmonary artery smooth muscle cells (PASMCs) plays an important role in the development of idiopathic pulmonary arterial hypertension (IPAH), whereas a rise in cytosolic Ca^{2+} concentration triggers PASM contraction and stimulates PASM proliferation. Recently, we demonstrated that upregulation of the TRPC6 channel contributes to proliferation of PASMCs isolated from IPAH patients. This study sought to identify single-nucleotide polymorphisms (SNPs) in the *TRPC6* gene promoter that are associated with IPAH and have functional significance in regulating TRPC6 activity in PASMCs.

Methods and Results—Genomic DNA was isolated from blood samples of 237 normal subjects and 268 IPAH patients. Three biallelic SNPs, -361 (A/T), -254 (C/G), and -218 (C/T), were identified in the 2000-bp sequence upstream of the transcriptional start site of TRPC6. Although the allele frequencies of the -361 and -218 SNPs were not different between the groups, the allele frequency of the -254 (C→G) SNP in IPAH patients (12%) was significantly higher than in normal subjects (6%; $P<0.01$). Genotype data showed that the percentage of -254 G/G homozygotes in IPAH patients was 2.85 times that of normal subjects. Moreover, the -254 (C→G) SNP creates a binding sequence for nuclear factor- κ B. Functional analyses revealed that the -254 (C→G) SNP enhanced nuclear factor- κ B-mediated promoter activity and stimulated TRPC6 expression in PASMCs. Inhibition of nuclear factor- κ B activity attenuated TRPC6 expression and decreased agonist-activated Ca^{2+} influx in PASMCs of IPAH patients harboring the -254 G allele.

Conclusions—These results suggest that the -254 (C→G) SNP may predispose individuals to an increased risk of IPAH by linking abnormal TRPC6 transcription to nuclear factor- κ B, an inflammatory transcription factor. (*Circulation*. 2009;119:2313-2322.)

Key Words: calcium ■ hypertension, pulmonary ■ ion channels ■ muscle, smooth ■ NF-kappa B

Pulmonary arterial hypertension (PAH) is a fatal and progressive disease characterized by elevated pulmonary vascular resistance resulting from severe pulmonary vascular remodeling.^{1–3} Approximately 6% of PAH patients have a family history of the condition and are referred to as having familial PAH; the rest are considered to have idiopathic PAH (IPAH). Although the cause of PAH remains unclear, elevated levels of mitogenic, angiogenic, and proinflammatory factors such as platelet-derived growth factor, endothelin-1,

interleukin-1 β /-6, soluble CD40 ligand, angiotensin-1, and serotonin have been reported to correlate with the onset of IPAH.^{2–4} Other factors associated with IPAH include the downregulation and dysfunction of voltage-gated K^+ channels⁵ and upregulation of the serotonin receptors and transporter.⁶ Moreover, mutations in the bone morphogenetic protein receptor-type II gene (*BMPR2*) have been demonstrated to associate with the development of familial PAH and IPAH.^{7,8} However, because *BMPR2* mutations are present in

Received March 25, 2008; accepted February 2, 2009.

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The online-only Data Supplement is available with this article at <http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.108.782458/DC1>.

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Circulation is available at <http://circ.ahajournals.org>

DOI: 10.1161/CIRCULATIONAHA.108.782458

only 15% to 20% of IPAH patients and the likelihood that clinical pulmonary hypertension will develop is only 10% to 20% in known carriers of *BMPT2* mutations,⁹ additional genetic and environmental factors other than *BMPT2* mutations may also contribute to the development of IPAH.

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Regardless of the initial pathogenic trigger, the elevated pulmonary vascular resistance and pulmonary arterial pressure in IPAH patients are caused mainly by sustained pulmonary vasoconstriction, concentric vascular remodeling, obliteration of small arteries and arterioles, in situ thrombosis, and formation of the plexiform lesion.^{1–3} Neointimal and medial hypertrophy in small and medium-sized pulmonary arteries is a key aspect of pulmonary vascular remodeling in IPAH patients and is attributed to excessive pulmonary artery smooth muscle cell (PASMC) proliferation.^{1,2}

Ca^{2+} operates as an important second messenger in cellular mechanisms leading to gene expression, cell proliferation, and contraction. A rise in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) in PASMCs is a major trigger for pulmonary vasoconstriction and an important stimulus for PASMC proliferation and migration.¹⁰ Conventional Ca^{2+} channel blockers (ie, nifedipine and diltiazem), which inhibit voltage-dependent Ca^{2+} channels in PASMCs, have been used to treat 15% to 20% of IPAH patients in clinical studies,¹¹ suggesting that increased $[\text{Ca}^{2+}]_{\text{cyt}}$ may be an important link in cellular pathways that lead to IPAH. Elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$ in PASMCs results from Ca^{2+} release from intracellular stores and Ca^{2+} influx through plasmalemmal Ca^{2+} channels.¹² In addition to voltage-dependent Ca^{2+} channels, it has been demonstrated that canonical transient receptor potential (TRPC) channels are responsible for Ca^{2+} entry in PASMCs.^{12–14}

TRPC6 is an important isoform of TRPC channels expressed in the lungs and pulmonary artery.^{12–15} We previously observed that TRPC6 mRNA and protein expression in lung tissues and PASMCs isolated from IPAH patients was substantially elevated compared with normal subjects and control patients with cardiopulmonary diseases.¹⁶ TRPC6 upregulation is also a critical initial step in the elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$ required for mitogen-mediated PASMC proliferation and a critical contributor to the elevated $[\text{Ca}^{2+}]_{\text{cyt}}$ in IPAH PASMCs.¹³ Downregulation of TRPC6 expression with siRNA significantly attenuates DNA synthesis and proliferation of PASMCs isolated from normotensive and IPAH patients.^{13,16} Together, these observations imply that upregulated *TRPC6* gene transcription may promote the development of IPAH.¹⁷

To test this hypothesis, we sequenced the 5'-regulatory region of *TRPC6* from 268 IPAH patients and identified a C to G (C→G) single-nucleotide polymorphism (SNP) at nucleotide –254 of the *TRPC6* gene that is associated with IPAH. Moreover, the –254C→G change creates a canonical nuclear factor- κ B (NF- κ B) binding site (GGGGGTCTCC) in the promoter region of *TRPC6* and significantly affects *TRPC6* gene transcription and TRPC6 channel function in PASMCs from IPAH patients who carry the –254G allele.

Methods

Subjects

A total of 237 normal subjects and 268 IPAH patients (including 124 patients enrolled at the University of California, San Diego Medical Center, 60 patients at the Vanderbilt University Medical Center, and 84 patients in the Giessen Lung Center in Germany) who participated in the study. All control subjects (all white) and IPAH patients were white (including 2 Hispanics) and were unrelated. The control subjects and patients were very closely matched racially and ethnically. We did not include data from blacks in this report because of the small sample number. The basic demographics of age, gender, and race in normal subjects and IPAH patients and the hemodynamics of all patients from each of the 3 centers are shown in Table I of the online-only Data Supplement. No significant difference was found ($P=0.68682$) between normal subjects (87.7 ± 9.35 [SD] mm Hg) and IPAH patients (88.3 ± 14.76 mm Hg) from whom we collected blood/DNA samples for this study. The diagnosis of IPAH was based on the criteria used in the National Institutes of Health Registry on Primary Pulmonary Hypertension. Informed consent was obtained from all subjects, and the study was approved by the Institutional Review Board at the University of California, San Diego.

Identification of SNP in the *TRPC6* Gene Promoter Region

Genomic DNA was extracted from the blood samples of normal subjects and patients with a Wizard genomic DNA purification kit. Six paired amplification polymerase chain reaction (PCR) primers (online-only Data Supplement Table II) were designed to amplify 6 overlapping DNA segments spanning 2000 bp upstream and 110 bp downstream of the transcriptional start site of human *TRPC6*. The purified PCR products were sequenced, analyzed by Chromas software, and compared with known SNPs deposited in the NCBI SNP databank (see supplementary Materials).

Cell Preparation and Culture

PASMCs from IPAH patients and non-pulmonary hypertensive (NPH) patients were isolated from lung tissues of transplant patients, and PASMCs from normal subjects were purchased from Lonza (Walkersville, Md). PASMCs were cultured in 5% CO_2 in air at 37°C in smooth muscle cell growth medium (Lonza) and used at the fourth to sixth passage.^{5,12} For tumor necrosis factor- α (TNF- α) stimulation experiments, the cells were growth arrested by culturing in smooth muscle cell basal medium (Lonza) for 24 hours before treatment.

Preparation of Cytoplasmic and Nuclear Extracts

Cytosolic and nuclear extracts from cultured PASMCs were collected using a modified protocol¹⁸ (see supplementary Materials).

Electrophoretic Mobility Shift Assay and Supershift Assay

Double-stranded oligonucleotide sequences from nucleotide –261 to –238 of *TRPC6* containing the –254C wild-type (5'-ATCCTCGCGGGGTCTCCTCGGCCT-3') or –254G mutated site (5'-ATCCTCGCGGGGTCTCCTCGGCCT-3') were synthesized and labeled by the biotin 3' end-labeling kit (Pierce Biotechnology Inc, Rockford, Ill). Each binding reaction (24°C for 40 minutes) contained 10 mmol/L Tris (pH 7.5), 50 mmol/L KCl, 1 mmol/L EDTA, 10 mmol/L dithiothreitol, 2.5% glycerol, 50 ng/mL poly(dI-dC), 5% albumin bovine, 4 μ g nuclear extract, and 40 to 50 fmol biotin end-labeled target DNA. Competition tests were used to verify whether the observed shifted bands were specific. The nuclear extract was preincubated with 200-fold excess unlabeled –254C or –254G probe before electrophoretic mobility shift assay. For supershift assays, polyclonal antibodies against p50 or p65 were added to the binding reaction before addition of the biotin end-labeled probe. The DNA-protein complexes were electrophoresed by 6% DNA retardation gels in 0.5× TBE running buffer and electrotransferred to nitrocellulose membranes. The biotin-labeled oligonucleotides in the

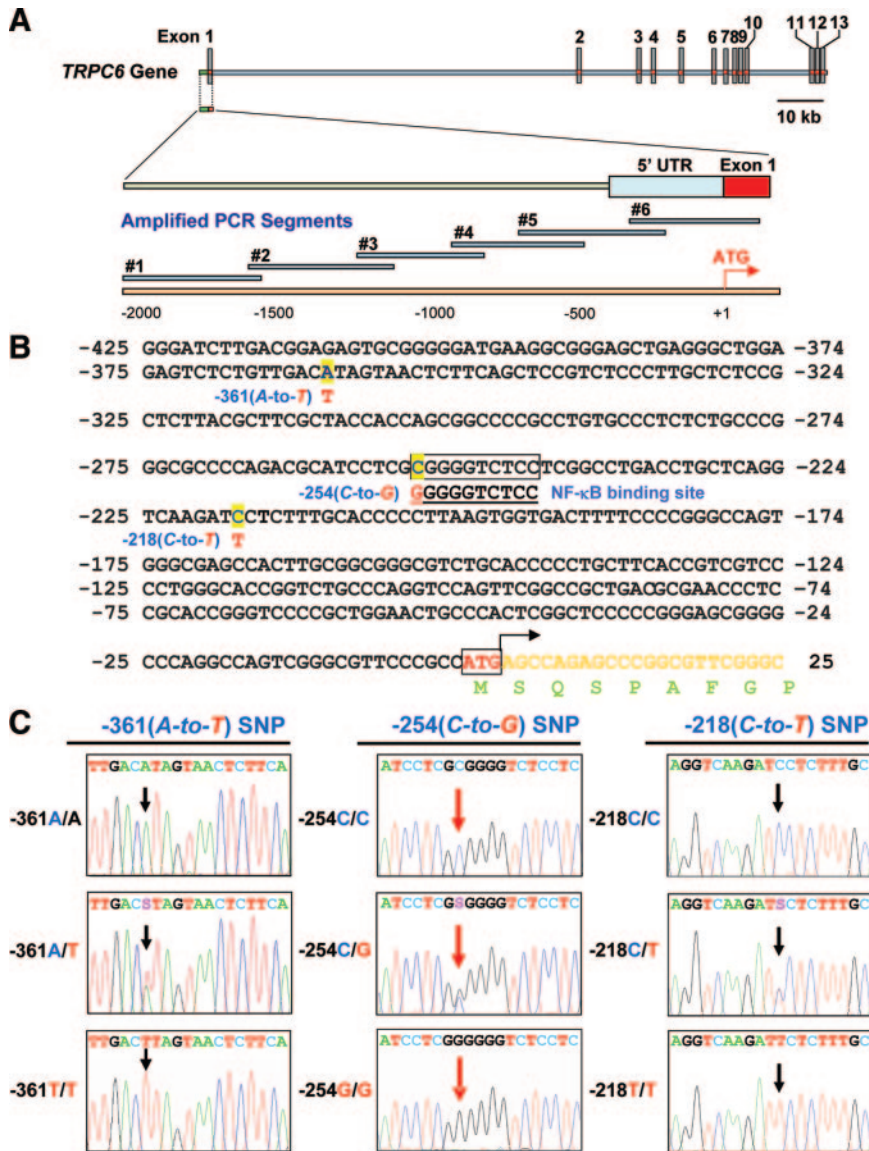


Figure 1. Identification of 3 SNPs in the 5'-regulatory region of the human *TRPC6* gene. A, Schematic diagram of the *TRPC6* gene and locations of the amplified PCR segments. B, The location of 3 SNPs, -361(A→T), -254(C→G), and -218(C→T), in the 5'-regulatory region of *TRPC6*. C, Representative sequence chromatographs for the 3 SNPs in IPAH patients. Arrow denotes position of the SNP. UTR indicates untranslated region.

membrane were detected with streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate (Pierce).

TRPC6 Putative Promoter Region Cloning and Promoter Activity

The human *TRPC6* putative promoter regions were amplified using genomic DNA isolated from PSMCs. The DNA fragments (-1682 to 110 bp) containing -254C or -254G were amplified by PCR. The PCR products were cloned into PCR 2.1 TOPO cloning vector. A transient expression system, pBlue-TOPO TA expression kit, was used to test the cloned *TRPC6* putative promoter activities. DNA fragments were fused to the promoterless β -glucuronidase (LacZ) reporter gene vector. COS-7 cells were transiently transfected with promoter vectors and, 24 hours after transfection, replated onto Petri dishes coated with poly-L-lysine. The promoter activities were detected and quantified by measuring the absorbance at 420 nm. Transfection efficiencies were normalized to green fluorescence protein (GFP) expression from a cotransfected pRL-CMV-GFP vector. The relative promoter activity is expressed as fold induction relative to the basal level of promoterless empty pBlue-TOPO vector. For LacZ staining, the cells were fixed with 0.05% glutaraldehyde and stained in X-Gal solution containing 40 mmol/L HEPES (pH 7.4), 5 mmol/L $K_3[Fe(CN)_6]$, 5 mmol/L $K_4[Fe(CN)_6]$, 2 mmol/L $MgCl_2$, 15 mmol/L NaCl, and 1 mg/mL X-Gal.

Generation of Recombinant Adenovirus Carrying Human *TRPC6*-Specific siRNA and Adenoviral Infection

Recombinant adenovirus carrying siRNA targeting human *TRPC6* was generated with an Invitrogen expression kit (Invitrogen, Carlsbad, Calif). For adenovirus infection experiments, PSMCs were infected with the appropriate virus in smooth muscle cell basal medium containing 0.2% FBS for 4 hours and used for experiments 24 to 48 hours after adenoviral infection (see supplementary Materials).

Human *TRPC6* cDNA Cloning and Transient Transfection

Human *TRPC6* cDNA was purchased from Open Biosystems (Huntsville, Ala). It was tagged with a hemagglutinin HA epitope sequence at the C terminal by introducing an in-frame HA epitope coding sequence before the stop codon and subcloning the corresponding cDNA into a mammalian expression pCDNA3 vector (Invitrogen) and pCMS-EGFP vector (BD Bioscience, San Jose, Calif), respectively. Resulting clones were confirmed by DNA sequencing and were designated pCDNA3-hTRPC6^{HA} and pCMS-EGFP-hTRPC6^{HA}. For transient transfection, electroporation-mediated transfection by nucleofector (Amax Biosystems, Walkersville, Md) was used, following the PSMC-specific protocol.

Table 1. Allele Frequency of the -254(C→G) SNP Is Significantly Higher in IPAH Patients Than in Normal Subjects

Position (nt.)	SNP	Allele	Normal Subjects (n=237), n (%)	IPAH (n=268), n (%)	Odds Ratio (95% CI)	P
-361	A to T	A	354 (74.7)	402 (75.0)	0.98 (0.74–1.31)	0.91
		T	120 (25.3)	134 (25.0)		
-254	C to G	C	443 (93.7)	473 (88.2)	1.97 (1.25–3.10)	0.0029
		G	30 (6.3)	63 (11.8)		

P values, odds ratios, and 95% CIs are calculated by χ^2 analysis for 2×2 contingency tables. The -361(A→T) and -218(C→T) SNPs are in complete linkage disequilibrium. Because values are identical, only the -361A→T SNP is shown for the sake of clarity.

Statistical Analysis

Values are expressed as mean±SEM. Statistical differences were assessed with unpaired Student *t* test or 1-way ANOVA with post hoc analysis. Differences were considered significant at values of $P < 0.05$. We used χ^2 analysis to compare the allele frequencies and genotype frequencies in normal subjects and patients. The odds ratio was estimated by the logistic regression model, assuming 95% as the CI, using the Woolf approximation. StatsDirect software (StatsDirect, Ltd, Cheshire, UK) was used for this analysis.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Three SNPs Were Identified in the Putative Promoter Region of *TRPC6*

The human *TRPC6* gene contains 13 exons (Figure 1A). Using specifically designed primers, we sequenced the 5'-untranslated region (-2000 bp upstream of the start codon, ATG) of *TRPC6* (Figure 1B) from 237 normal subjects and 268 IPAH patients. DNA sequence alignment with the human genomic *TRPC6* sequence (AP003080) revealed 3 variants, -361(A→T), -254(C→G), and -218(C→T) (Figure 1B and 1C). All 3 SNPs have been reported and are listed in the NCBI SNP database: -254(C→G) SNP matches to rs3824934, -361(A→T) to rs41302375, and -218(C→T) to rs56134796. The -361(A→T) and -218(C→T) SNPs are in complete linkage disequilibrium.

Allele and Genotype Frequencies of the -254C→G SNP in *TRPC6* Are Significantly Higher in IPAH Patients Than in Normal Subjects

To examine whether the 3 SNPs of *TRPC6* are associated with IPAH, we analyzed their allele frequencies and genotype frequencies in IPAH patients and normal control subjects. As shown in Table 1, the allele frequency of the -254G SNP was significantly higher in IPAH patients (12%) than in normal subjects (6%; $P < 0.01$), whereas the allele frequencies of the -361(A→T) and -218(C→T) SNPs were comparable ($P = 0.83$) between IPAH patients (25% and 25%) and normal subjects (25% and 25%). Both the homozygous and heterozygous variants of the -254(C→G) SNP were identified in IPAH patients (Figure 1C). Genotype analysis showed that 25 of 237 normal subjects (10.5%) were heterozygotes and 3 of 237 of normal subjects (1.3%) were homozygotes for the -254(C→G) SNP. In contrast, 43 of 268 of the IPAH patients (16.0%) were heterozygotes (-254C/G) and 10 of

Table 2. The Homozygous -254G/G Genotype Has a Significantly Higher Frequency in IPAH Patients Than in Normal Subjects

SNP	Genotype	Normal (n=237), n (%)	IPAH (n=268), n (%)	Odds Ratio (95% CI)	P
-361(A→T)	-361 A/A	132 (55.7)	152 (56.7)	0.96* (0.67–1.36)	0.82*
	-361 A/T	90 (38.0)	98 (36.6)		
	-361 T/T	15 (6.3)	18 (6.7)		
-254(C→G)	-254 C/C	209 (88.2)	215 (80.2)	1.84† (1.12–3.02)	0.015†
	-254 C/G	25 (10.5)	43 (16.0)		
	-254 G/G	3 (1.3)	10 (3.7)		

P values, odds ratios, and 95% CIs are calculated by χ^2 analysis for comparison of the genotype frequencies between IPAH patients and normal subjects.

*Odds ratio and P value for (-361 A/T and -361 T/T) vs -361A/A and for (-218 C/T and -218 T/T) vs -218C/C.

†Odds ratio and P value for (-254 C/G and -254 G/G) vs -254 C/C. The -361(A→T) and -218(C→T) SNPs are in complete linkage disequilibrium. Because their allele frequencies, odds ratio, and P values are identical, only the -361A→T SNP is shown for the sake of clarity.

268 patients (3.7%) were homozygotes (-254G/G) ($P < 0.02$ between normal subjects and IPAH patients for both heterozygotes and homozygotes; Table 2). The -254C/G heterozygote and -254G/G homozygote frequencies in IPAH patients were 1.52 (16.0% versus 10.5%) and 2.85 (3.7% versus 1.3%) times, respectively, those of normal subjects ($P < 0.02$). A full description of the allele frequencies for each patient cohort is provided in online-only Data Supplement Table III.

The -254(C→G) SNP Generates an NF- κ B Binding Site at the Promoter Region of *TRPC6*

Interestingly, the C-to-G conversion at nucleotide -254 results in the sequence alteration of "CGGGGTCTCC" (nucleotide -254 to -245) to "GGGGGTCTCC," which matches the sequence of "GGGRNYYCC" (where R=A or G; Y=C or T; N=any nucleotide), a consensus sequence for the transcription factor NF- κ B. Therefore, the -254(C→G) SNP adds a putative NF- κ B binding site into the 5'-untranslated region of *TRPC6*.

We performed an electrophoretic mobility shift assay to determine whether the -254(C→G) SNP confers functional NF- κ B protein binding to the promoter region of *TRPC6*. Biotin-labeled oligoduplex probes with nucleotides encompassing sequences between nucleotide -238 and -261 containing -254C (-254C probe) or -254G (-254G probe) were incubated with nuclear extracts isolated from normal PSMCs. To evaluate the physiological or functional consequences, PSMCs were stimulated with TNF- α before the electrophoretic mobility shift assay was performed. As shown in Figure 2A, TNF- α (10 ng/mL) stimulated translocation of the p50 and p65 of the NF- κ B components into the nucleus of PSMCs, indicating that TNF- α is an effective stimulator for nuclear translocation of NF- κ B.

Two DNA-bound NF- κ B complexes were identified with the -254G probe (designated C1 and C2), whereas NF- κ B did not bind to the -254C probe (Figure 2B, left). Complex formation (as indicated by C1 and C2) was inhibited by the addition of excess unlabeled -254G probe but not by

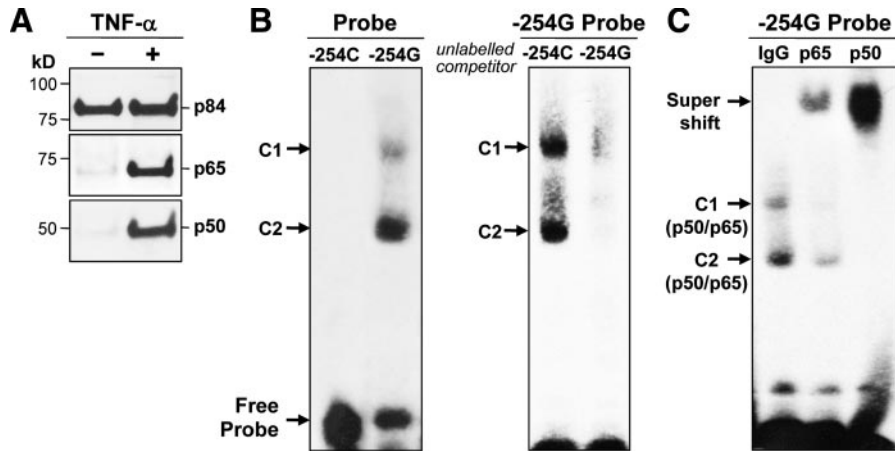


Figure 2. Nuclear NF- κ B proteins of PASMCs specifically bind to the -254(C \rightarrow G) SNP-generated NF- κ B binding site. A, NF- κ B activation demonstrated by translocation of p50 and p65 from the cytoplasm to the nucleus. PASMCs from normal subjects were stimulated with (+) or without (-) TNF- α (10 ng/mL). B, Electrophoretic mobility shift assay and competition analysis. TNF- α -stimulated nuclear extracts from PASMCs were incubated with biotin-labeled double-stranded oligonucleotides corresponding to the 24-bp sequences containing the wild-type -254C or mutant -254G probes. Nuclear extract/oligonucleotide complexes (C1 and C2) are indicated by arrows. C, Antibody-mediated supershift assay. NF- κ B p50 and p65 antibodies identified complexes C1 as p50/p65 and C2 as p50/p50 NF- κ B-containing complexes.

unlabeled -254C probe because the unlabeled -254G probe competitively bound to the same DNA binding site as NF- κ B, whereas the unlabeled -254C probe failed to compete for binding to the NF- κ B binding site. These results indicate that NF- κ B specifically binds to the -254G probe (Figure 2B, right). Supershifted complexes were detected by the addition of the anti-p65 or anti-p50 antibody to the binding reaction, indicating

that the NF- κ B C1 and C2 complexes contain a p50/p65 heterodimer and a p50/p50 homodimer, respectively (Figure 2C).

The -254(C \rightarrow G) SNP Affects NF- κ B-Mediated TRPC6 Promoter Activity

Gene constructs were generated to define whether the -254(C \rightarrow G) SNP introduces an active NF- κ B-regulated

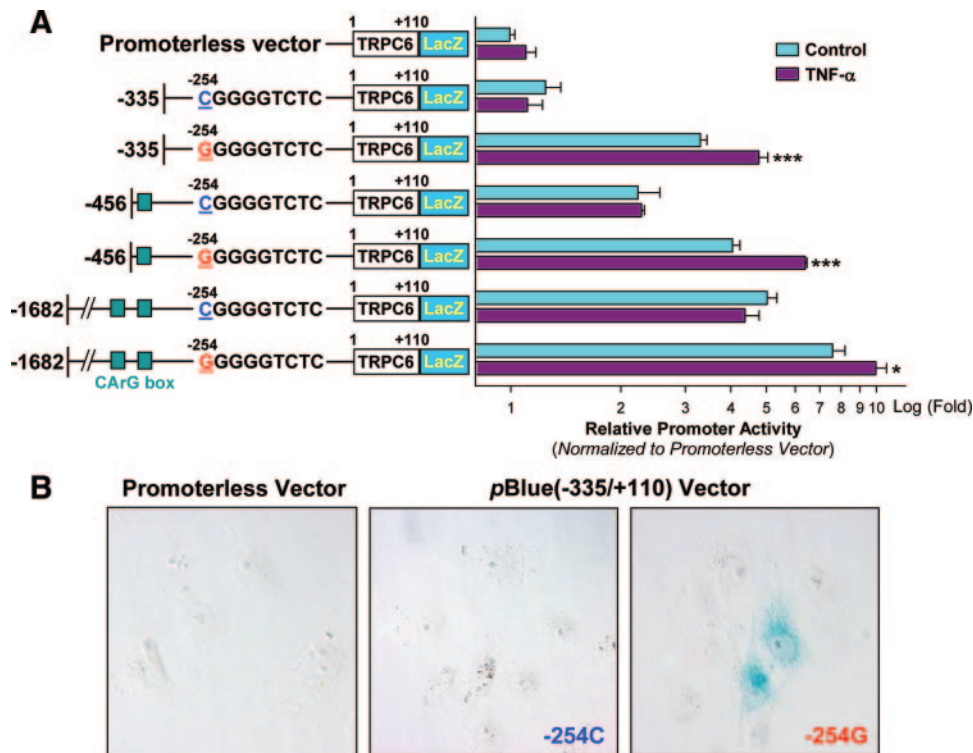


Figure 3. The basal and TNF- α -mediated promoter activities are enhanced in PASMCs transfected with the reporter constructs containing -254G allele. A, Schematic diagram (left) of the reporter constructs used for the promoter assay and the relative promoter activity in control and TNF- α -treated cells (right). The relative β -galactosidase activity (right) represents the mean \pm SE (plotted on a log scale) of 3 independent experiments performed in quadruplicate. * P <0.05, *** P <0.001 vs control cells. B, COS-7 cells transfected with pBlue(-335/+110) constructs that contain -254C or -254G allele. X-Gal staining was used to detect the β -galactosidase-stained cells. Empty (or promoterless) pBlue TOPO vector was used as control.

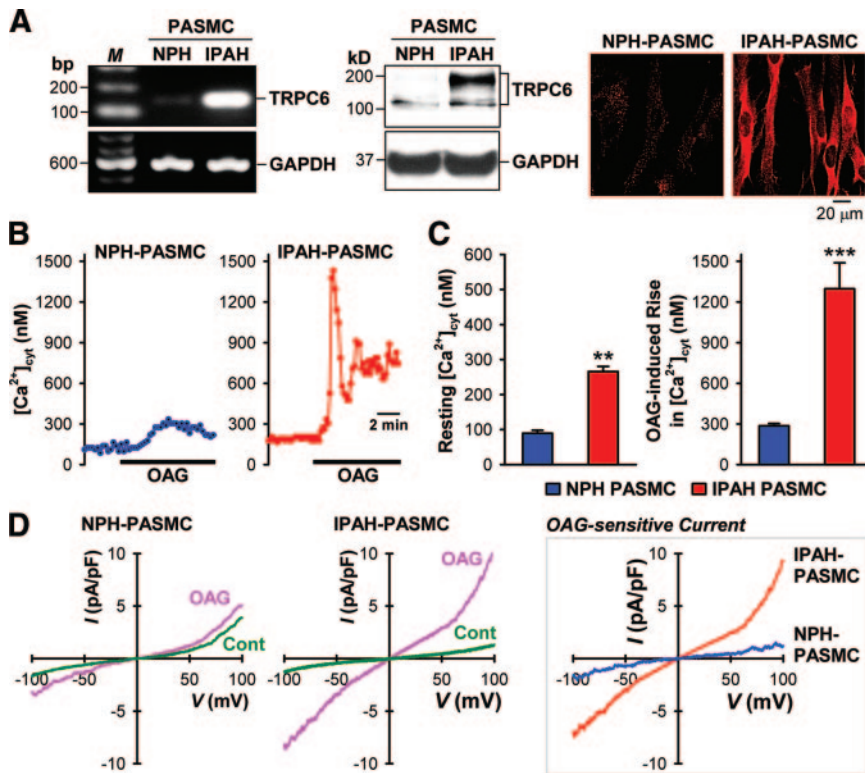


Figure 4. Upregulated TRPC6 expression in PSMCs from IPAH patients increases the resting $[Ca^{2+}]_{cyt}$ and enhances agonist-mediated Ca^{2+} influx. A, mRNA and protein expression of TRPC6, determined by RT-PCR (left), Western blot (middle), and immunocytochemistry (right), respectively, is significantly higher in IPAH PSMCs than in control PSMCs from NPH patients. B, Representative records of $[Ca^{2+}]_{cyt}$ changes in response to OAG (100 μmol/L) in control (NPH) and IPAH PSMCs. C, Summarized data of the resting $[Ca^{2+}]_{cyt}$ and the amplitude of the OAG-induced increase in $[Ca^{2+}]_{cyt}$ in NPH and IPAH PSMCs. ** $P < 0.01$, *** $P < 0.001$ vs NPH PSMCs. D, Representative currents recorded in NPH (left) and IPAH (middle) PSMCs before (Cont) and during (OAG) application with OAG (100 μmol/L). OAG-sensitive currents, generated by subtracting the currents recorded during OAG from the control currents, in NPH and IPAH PSMCs are shown in the right panel.

promoter region in the *TRPC6* gene. Three different-length constructs of the 5' upstream region of *TRPC6* containing -254C or -254G were cloned and inserted into a promoterless *pBlue-TOPO* vector upstream of a β -glucuronidase (*LacZ*) reporter gene. The resulting constructs were designated *pBlue*(-335/+110), *pBlue*(-456/+110), and *pBlue*(-1682/+110), respectively.

In COS-7 cells transiently transfected with these vectors, TNF- α (20 ng/mL for 24 hours), by causing nuclear translocation of NF- κ B (p65 and p50), significantly enhanced promoter activity for the constructs [*pBlue*(-335/+110), $P < 0.001$; *pBlue*(-456/+110), $P < 0.001$; *pBlue*(-1682/+110), $P = 0.027$] with the -254G mutation but negligibly affected promoter activity for the constructs with the -254C wild-type sequence (Figure 3A) [$P = 0.437$ (-335/+110), $P = 0.889$ (-456/+110), $P = 0.231$ (-1682/+110)]. Compared with cells transfected with promoterless *pBlue-TOPO* vector, the basal promoter activity (or the relative induction of β -galactosidase activity in cells not treated with TNF- α) significantly increased when the 5' upstream region was extended from nucleotide -335 to -456 and -1682 in the wild-type -254C template (Figure 3A). X-Gal staining confirmed these results by exhibiting an intense staining corresponding to β -galactosidase in the endoplasmic reticulum with the -254G *pBlue*(-335/+110) construct but not with the -254C construct (Figure 3B). Together, these data indicate that the -254(C→G) SNP modulates *TRPC6* gene promoter activity and enhances TNF- α - and NF- κ B-mediated *TRPC6* transcription.

Upregulated TRPC6 Expression Contributes to Regulating $[Ca^{2+}]_{cyt}$ in PSMCs of IPAH Patients

We previously reported that TRPC6 expression was significantly increased in IPAH PSMCs compared with normal

PASMCs. To examine whether upregulated TRPC6 mRNA and protein expression in IPAH PSMCs (with the -254G allele) (Figure 4A) contributes to regulating $[Ca^{2+}]_{cyt}$, we measured and compared the resting $[Ca^{2+}]_{cyt}$ and agonist-mediated Ca^{2+} influx in normal PSMCs and IPAH PSMCs. The resting $[Ca^{2+}]_{cyt}$ and the increase in $[Ca^{2+}]_{cyt}$ induced by 1-oleoyl-2-acetyl-*sn*-glycerol (OAG; 100 μmol/L), a membrane-permeable diacylglycerol analog known to activate TRPC6 channels, were both significantly enhanced in IPAH PSMCs compared with normal PSMCs ($P < 0.001$ for resting and OAG-induced $[Ca^{2+}]_{cyt}$; Figure 4B and 4C). The OAG-induced increase in whole-cell cation currents in IPAH PSMCs was also significantly enhanced compared with normal PSMCs (Figure 4D). Inhibition of TRPC6 expression in IPAH PSMCs using siRNA markedly attenuated the OAG-mediated increase in $[Ca^{2+}]_{cyt}$ ($P < 0.001$) and reduced the resting $[Ca^{2+}]_{cyt}$ ($P = 0.003$; Figure 5). In normal PSMCs (with the -254C/C genotype), overexpression of the human *TRPC6* gene significantly enhanced the OAG-induced increase in $[Ca^{2+}]_{cyt}$ ($P < 0.001$; Figure 6) but not resting $[Ca^{2+}]_{cyt}$ ($P = 0.075$). These results indicate that upregulated TRPC6 expression is functionally involved in regulating resting $[Ca^{2+}]_{cyt}$ and agonist-mediated Ca^{2+} entry in PSMCs from IPAH patients harboring the -254G allele.

Inhibition of NF- κ B Activity by I κ B α Attenuates TRPC6 Expression and Function in PSMCs From IPAH Patients With the -254G Allele

I κ B α is an inhibitory subunit of the NF- κ B complex that binds to p50/p65 and keeps the complex in the cytoplasm. I κ B α superrepressor is a nondegradable mutant dominant-negative variant of I κ B α that inhibits NF- κ B activation.¹⁹ Overexpression of the I κ B α superrepressor with an adenovi-

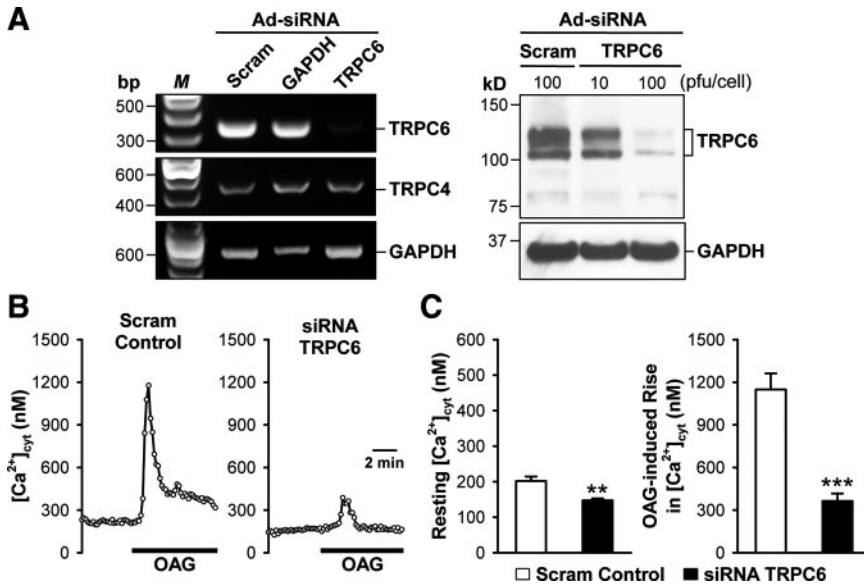


Figure 5. Downregulation of TRPC6 with siRNA inhibits OAG-induced Ca^{2+} influx in PSMCs from IPAH patients. A, mRNA (left) and protein (right) expression levels of TRPC6, TRPC4, and GAPDH are shown in IPAH PSMCs treated with scrambled siRNA, GAPDH siRNA, or TRPC6 siRNA. B, Representative records of $[Ca^{2+}]_{cyt}$ changes in response to OAG in IPAH PSMCs treated with scrambled siRNA (left) or TRPC6 siRNA (right). C, Summarized data of the resting $[Ca^{2+}]_{cyt}$ and OAG-induced $[Ca^{2+}]_{cyt}$ increases (right) in IPAH PSMCs treated with scrambled or TRPC6 siRNA. ** $P < 0.01$, *** $P < 0.001$ vs scrambled siRNA-treated control cells.

ral vector markedly inhibited translocation of p50 and p65 into the nucleus in PSMCs from IPAH patients (with the -254G allele) and significantly inhibited TNF- α -mediated upregulation of TRPC6 (Figure 7A). Western blot analysis indicated 2 bands corresponding to TRPC6 protein, 1 at ≈ 110 kDa and the other at 130 kDa. The ≈ 110 -kDa band reflects nascent TRPC6 early in the secretory pathway, whereas the 130-kDa band reflects complex-glycosylated TRPC6 that appears to maintain a relatively long half-life.²⁰ In IPAH PSMCs (-254G allele) treated with TNF- α , inhibition of

NF- κ B by overexpression of the I κ B α superrepressor predominantly downregulated the nascent core-glycosylated isoform (the ≈ 110 -kDa band) of TRPC6 (Figure 7A, right), implying that the effect is due to a reduction in *TRPC6* gene transcription. Together, these data support the contention that NF- κ B plays an important role in regulating *TRPC6* gene transcription and expression in IPAH patients harboring the -254(C \rightarrow G) SNP. This finding is supported by similar assays performed in PSMCs from -254C/C IPAH. Unlike the -254C/G individuals, TRPC6 protein expression was not

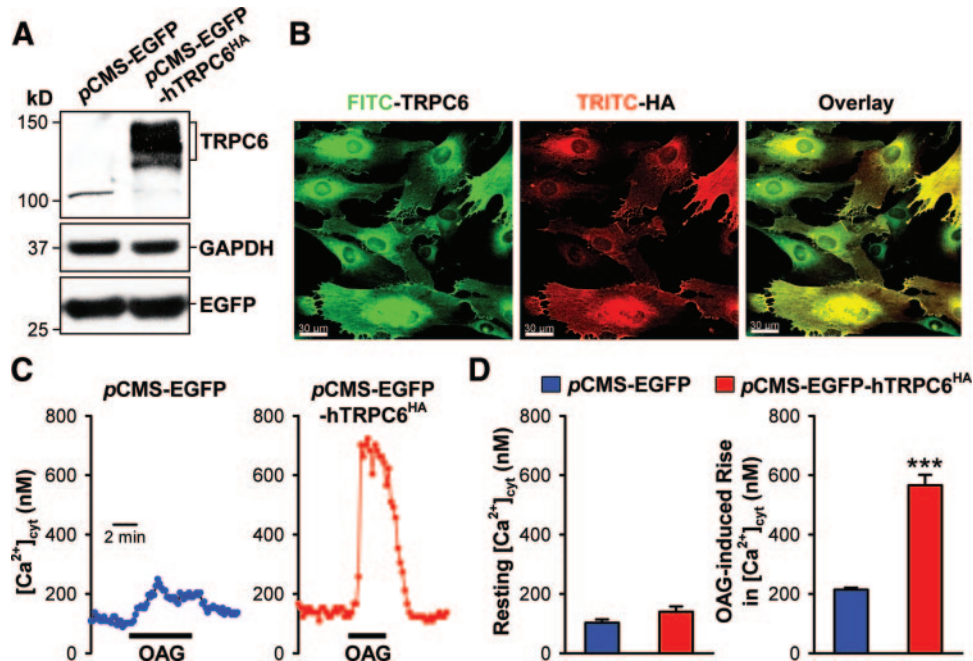


Figure 6. Overexpression of TRPC6 in normal PSMCs (with -254C/C genotype) enhances the OAG-induced increase in $[Ca^{2+}]_{cyt}$. A, Western blot analysis of TRPC6 in cells transfected with pCMS-EGFP or pCMS-EGFP-hTRPC6^{HA}. B, Exogenous human TRPC6^{HA} expression was identified by immunofluorescence staining. Normal PSMCs were transfected with pcDNA3-hTRPC6^{HA}. HA epitope tag was stained by TRITC-conjugated anti-HA monoclonal antibody. TRPC6 antibody, followed by FITC-conjugated secondary antibody, was used to detect TRPC6 expression. C, Representative records of $[Ca^{2+}]_{cyt}$ changes in response to OAG in PSMCs transfected with pCMS-EGFP or pCMS-EGFP-hTRPC6^{HA}. D, Summarized data of the resting $[Ca^{2+}]_{cyt}$ and the amplitude of the OAG-induced $[Ca^{2+}]_{cyt}$ increases in cells transfected with pCMS-EGFP or pCMS-EGFP-hTRPC6^{HA}. *** $P < 0.001$ vs pCMS-EGFP cells.

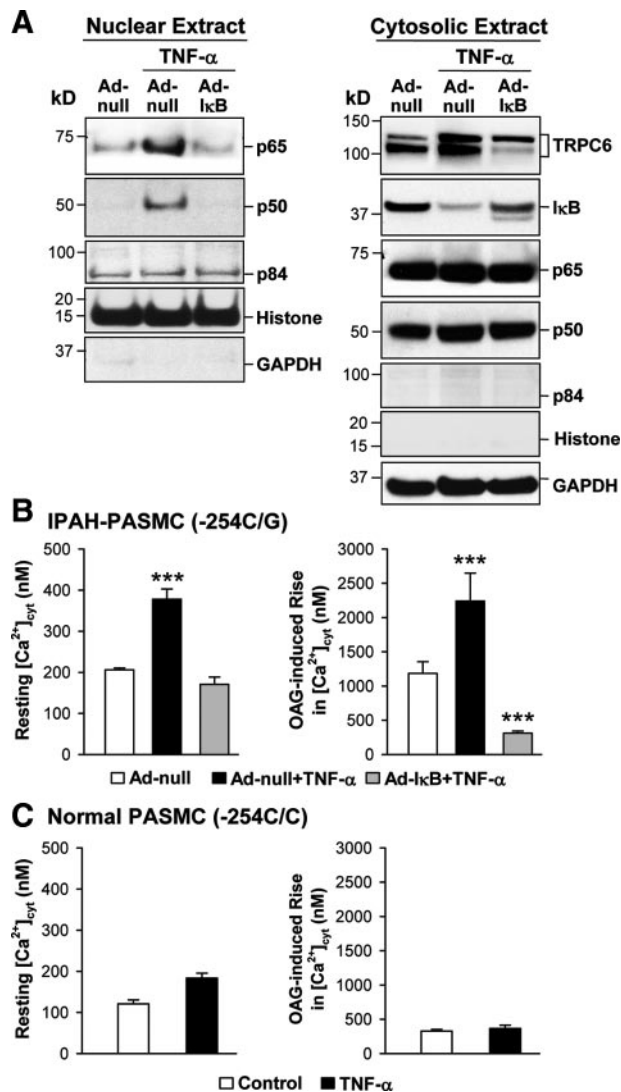


Figure 7. Overexpression of the inhibitory subunit IκBα attenuates NF-κB-mediated *TRPC6* expression and inhibits agonist-mediated Ca^{2+} influx in IPAH-PASMC. **A**, Overexpression of IκBα (Ad-IκB) inhibits translocation of NF-κB p50 and p65 into the nucleus (left) and diminishes TNF-α-stimulated *TRPC6* expression (right). GAPDH, histone (H2A), and p84 antibodies were used as controls for nuclear and cytoplasmic proteins. Experiments were reproduced 3 times. **B**, Summarized results showing that TNF-α increases the resting $[Ca^{2+}]_{cyt}$ (left) and enhanced OAG-induced $[Ca^{2+}]_{cyt}$ rise (right) in IPAH PASMCs (–254C/G), whereas IκBα (Ad-IκB) abolishes the TNF-α-mediated enhancement of the resting $[Ca^{2+}]_{cyt}$ and OAG-mediated Ca^{2+} influx. **C**, Summarized results showing that TNF-α negligibly affects the resting $[Ca^{2+}]_{cyt}$ (left) and OAG-induced $[Ca^{2+}]_{cyt}$ increase (right) in normal PASMCs (–254C/C).

significantly altered in –254C/C PASMCs treated with TNF-α (see online-only Data Supplement Figure I).

To determine whether the enhanced basal *TRPC6* expression and TNF-α-mediated *TRPC6* upregulation in the –254C/G variant have functional significance, we compared the levels of resting $[Ca^{2+}]_{cyt}$ and OAG-induced Ca^{2+} influx in unstimulated and TNF-α-stimulated –254C/G PASMCs from IPAH patients to the levels in PASMCs from –254C/C normal subjects. As shown in Figure 7B and 7C, the resting $[Ca^{2+}]_{cyt}$ and the OAG-induced $[Ca^{2+}]_{cyt}$ increase in unstimu-

lated –254C/G IPAH PASMCs were both significantly greater than in –254C/C normal PASMCs. TNF-α treatment significantly increased the resting $[Ca^{2+}]_{cyt}$ and enhanced the OAG-induced $[Ca^{2+}]_{cyt}$ increase in PASMCs from –254C/G IPAH patients (Figure 7B) but not in normal PASMCs from –254C/C subjects ($P=0.424$; Figure 7C). Furthermore, overexpression of IκBα abolished the TNF-α-mediated increase in the resting $[Ca^{2+}]_{cyt}$ and enhancement of the OAG-mediated Ca^{2+} influx ($P<0.001$) in PASMCs from –254C/G IPAH patients (Figure 7B). These results provide strong evidence that the –254(C→G) SNP in the *TRPC6* gene has functional significance in regulating basal $[Ca^{2+}]_{cyt}$ and agonist-mediated Ca^{2+} entry in PASMCs.

Discussion

We report 4 primary findings in this study: (1) A “gain-in-function” –254(C→G) SNP in *TRPC6* is associated with IPAH; (2) the –254(C→G) SNP generates an active NF-κB binding site that regulates *TRPC6* transcription; (3) nuclear translocation of NF-κB upregulates *TRPC6* expression and enhances agonist-induced Ca^{2+} influx in IPAH PASMCs with the –254G allele; and (4) inhibition of nuclear translocation of NF-κB attenuates *TRPC6* expression and function in PASMCs from IPAH patients harboring a –254G allele.

Pulmonary arterial neointimal and medial hypertrophy is a major pathological feature in IPAH.^{1–3} One of the important mechanisms in pulmonary vascular wall thickening is increased PASMC proliferation and migration resulting from a rise in $[Ca^{2+}]_{cyt}$.^{12–16} As a critical signaling element, intracellular Ca^{2+} is responsible for activating multiple signal transduction cascades that trigger PASMC contraction, activate gene expression, and stimulate PASMC proliferation.^{12–16,21} Upregulated *TRPC6* plays an important role in the development of pulmonary vascular remodeling in IPAH patients.¹⁶

TRPC6 is a canonical transient receptor potential channel isoform believed to be involved in forming receptor-operated Ca^{2+} channels.^{17,22,23} Studies on *TRPC6* knockout mice revealed that *TRPC6* plays a central role in receptor-operated control of vascular smooth muscle tone.²⁴ In cardiac myocytes, overexpressed *TRPC6* plays an important role in forming a Ca^{2+} -dependent calcineurin-NFAT-*TRPC6* loop that leads to pathological cardiac hypertrophy and remodeling.²⁵ In PASMCs, *TRPC6* is involved in forming functional Ca^{2+} channels that are activated by vasoconstrictor and mitogenic factors.^{13,14,26} Downregulation of *TRPC6* inhibits PASMC proliferation.^{13,16}

Compared with normal subjects and normotensive patients, mRNA and protein expression levels of *TRPC6* in lung tissue and PASMCs were significantly higher in IPAH patients, suggesting an abnormal *TRPC6* gene transcription in these patients.¹⁶ In the present study, we found that the allele frequency of the –254(C→G) SNP in *TRPC6* was significantly higher in IPAH patients than in normal subjects. In contrast, the allele frequencies of the 2 neighboring SNPs, –361(A→T) and –218(C→T), were comparable between normal subjects and IPAH patients. Moreover, 3.7% of IPAH patients possess a homozygous –254G/G genotype, whereas 1.3% of the normal subjects have the homozygous –254G/G genotype. These data imply that the –254(C→G) SNP may

be responsible for the abnormal TRPC6 expression and function in a subpopulation of IPAH patients.

The C→G conversion in IPAH patients harboring the −254(C→G) SNP introduces a known NF-κB binding site (GGGGGTCTCC)²⁷ in the 5'-regulatory region of *TRPC6* and confers NF-κB-mediated transcriptional activation of *TRPC6*. The −254G-generated NF-κB binding sequence in IPAH PSMCs showed measurable binding affinity for the p50/p50 homodimer and p50/p65 heterodimer of the NF-κB complex. Furthermore, TNF-α-mediated activation of NF-κB significantly upregulated TRPC6 expression, increased the resting $[Ca^{2+}]_{\text{cyt}}$, and enhanced the agonist-induced Ca^{2+} influx in PSMCs from IPAH patients with the −254(C→G) SNP. Inhibition of NF-κB with a mutant dominant-negative IκBα markedly attenuated TNF-α-induced enhancement of TRPC6 expression, resting $[Ca^{2+}]_{\text{cyt}}$, and OAG-induced $[Ca^{2+}]_{\text{cyt}}$ rise in IPAH PSMCs with the −254G allele. These results support a functional role for the −254(C→G) SNP in NF-κB-induced *TRPC6* gene transcription and TRPC channel activity.

In addition, our observations showed that the −254(C→G) SNP significantly increased the basal promoter activity of *TRPC6* that is not related to NF-κB. The increase in basal promoter activity occurred when the pBlue(−335/+110) construct was used and was maintained when the pBlue(−456/+110) and pBlue(−1682/+110) constructs were used. These results suggest that the −254(C→G) SNP not only enhances NF-κB-mediated *TRPC6* transcription but also may enhance basal transcription of *TRPC6* in PSMCs. Because many binding sites are present in the promoter region of *TRPC6*, it is possible that the −254G-generated NF-κB binding site may facilitate interactions of various transcription factors (eg, NFAT, AP-1) to increase *TRPC6* transcription.²⁵

The pathophysiological significance of insertion of an NF-κB binding site into the *TRPC6* gene may underlie the potential linkage of immune or inflammatory responses to the upregulation of TRPC6 channels in the pulmonary vasculature. The NF-κB transcription factor family plays an essential role in immune and inflammatory responses and maintains an antiapoptotic function in normal and malignant cells.^{28,29} Given the fact that viral and bacterial infection, along with the inflammatory response resulting from the infection, is related to the development of the plexiform lesion and vascular remodeling in IPAH patients,^{30,31} the −254(C→G) SNP in *TRPC6* may serve as an important genetic variation that links inflammatory response to the occurrence of IPAH and predisposes the −254G allele carriers to an increased risk for inflammation-mediated pulmonary arteriopathy. Furthermore, a potential exists that the −254(C→G) SNP in *TRPC6* may portend more importantly for patients with PAH secondary to connective tissue diseases (eg, scleroderma), HIV/AIDS, and schistosomiasis, who bear a significant inflammatory burden. Further study is needed to define whether the −254(C→G) SNP in *TRPC6* (or other genetic variations in genes involved in the NF-κB pathway) may be an important factor in separating the scleroderma (or HIV/AIDS or schistosomiasis) patients who develop pulmonary hypertension from those who do not.

IPAH appears to have a heterogeneous origin involving multiple genetic, molecular, and cellular abnormalities.^{2,3,32} SNPs in genes encoding *BMPR2*,^{7–9} activin receptor-like

kinase 1 (*ALK1*),³³ and serotonin transporter (*5-HTT*)⁶ have been linked to familial PAH and IPAH. Our data also link the −254(C→G) SNP in *TRPC6* to IPAH and indicate that the heterozygous −254C/G and homozygous −254G/G genotypes are associated with the occurrence of IPAH. However, mutations or SNPs in all these genes have been found in only a small portion of IPAH patients. It seems that the abnormality in each of the genes is important by itself, but none of them is sufficient to cause the disease. Therefore, the causal and pathogenic mechanisms of IPAH may involve abnormalities in multiple genes and gene products. It may be explained by the notion of multiple-hit theory proposed by many investigators.^{2,34,35} For instance, inheritance of mutations in *TRPC6* and other genes (eg, *BMPR2*, *5-HTT*, *ALK1*), followed by exposure to viral infection,^{31,36} inflammatory factors,^{4,37} and anorexic drugs or anorexigens,³⁸ results in pathogenic changes in the pulmonary vasculature and the development of IPAH.

Conclusions

We have identified a unique genetic variation, the −254(C→G) SNP in *TRPC6*, which may link the inflammatory response to upregulation of TRPC6, aberrant regulation of cytoplasmic Ca^{2+} in PSMCs, and ultimately alterations in the pulmonary vasculature. The enhanced transcriptional regulation of *TRPC6* and augmented function of TRPC6 channels resulting from the −254(C→G) SNP may predispose individuals who have this mutation to an increased risk of developing IPAH.

Acknowledgments

We thank Dr J.A. Kozak for helpful discussions and advice on the electrophysiological recording of TRPC6 currents and Drs H. Gall, S.S. Pullamsetti, H.T. Mueller, and T. Stoecker for technical assistance.

Sources of Funding

This work was supported in part by the American Heart Association (0630117N to Dr Yu) and the National Institutes of Health (HL64945, HL66012, and HL54043 to Dr Yuan; NS14609 to Dr Cahalan).

Disclosures

None.

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CLINICAL PERSPECTIVE

Genetic modifications of key genes have been associated with increased pulmonary vascular remodeling and incidence of disease in idiopathic pulmonary arterial hypertension (IPAH). We previously reported that upregulation of canonical transient receptor potential 6 (TRPC6) may be responsible for the abnormal pulmonary artery smooth muscle cell proliferation and pulmonary vascular medial hypertrophy in IPAH patients. This study identifies a gain-in-function single-nucleotide polymorphism (SNP) in the *TRPC6* promoter −254(C→G) with a significantly higher allele frequency in IPAH patients from the USA and Germany. This −254(C→G) variation generates a functional binding site for nuclear factor- κ B, which results in regulation of TRPC6 expression and function in modulating cytosolic Ca^{2+} levels in pulmonary artery smooth muscle cells. Examination of isolated pulmonary artery smooth muscle cells from IPAH patients further confirms that the presence of the −254(C→G) single-nucleotide polymorphism is linked to TRPC6 expression and function. These findings provide a strong putative link between the inflammatory response (or activated nuclear factor- κ B), which has been suggested in part to underlie IPAH, and altered TRPC6 channel function, providing an alternative therapeutic strategy to treat IPAH patients and to prevent the occurrence of IPAH.

A Functional Single-Nucleotide Polymorphism in the *TRPC6* Gene Promoter Associated With Idiopathic Pulmonary Arterial Hypertension

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Circulation. 2009;119:2313-2322; originally published online April 20, 2009;
doi: 10.1161/CIRCULATIONAHA.108.782458

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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SUPPLEMENTAL MATERIAL

Determination of pulmonary hemodynamics. A flow directed, balloon tipped Swan-Ganz catheter was positioned into right ventricle and then pulmonary artery via the internal jugular vein. Hemodynamic measurements, such as mean PAP, were obtained by a pressure transducer (Namic/Boston Scientific, Natick, MA) connected to a Mac-Laboratory 7000 hemodynamic and electrocardiographic monitoring system (GE Medical System, Waukesha, WI). Cardiac output (CO) was measured by a thermodilution technique, and PVR was calculated according to the measured mean PAP, pulmonary artery wedge pressure (P_{wedge}) and CO by the monitoring system.

Supplementary Table 1. Demographic and hemodynamic characteristics of normal subjects and IPAH patients

Characteristic	Summary		Normal Subjects			IPAH Patients		
	NPH	IPAH	UCSD	Giessen	Vanderbilt	UCSD	Giessen	Vanderbilt
Number of Subjects	237	268	127	110	N.A.	124	84	60
Sex (No.)								
Female	138	205	71	67	N.A.	96	60	50
Male	99	63	56	43		28	24	10
Race								
White	237	268	127	110	N.A.	124	84	60
Age (yr)	47.9±1.7	47.9±0.9	54.5±1.4	40.3±1.7		44.9±1.4	50.2±1.7	51.2±1.7
Female	48.6±1.7	48.4±1.0	58.5±1.9	38.2±2.1	N.A.	45.8±3.0	50.7±1.9	53.1±1.8
Male	46.8±1.5	46.4±2.0	49.3±1.5	43.6±2.8		44.5±1.3	49.0±3.5	41.9±3.9
Mean PAP (mmHg)								
Female	N.A.	55.3±14.4	N.A.	N.A.	N.A.	55.6±1.3	54.3±1.8	55.8±1.4
Male		54.3±0.9				54.8±1.4	52.3±1.9	55.8±1.5
		58.3±2.2				58.3±2.9	59.3±4.1	55.5±4.0
PVR (dyn·min ⁻¹ ·cm ⁻⁵)								
Female	N.A.	1063.4±34.8	N.A.	N.A.	N.A.	999.7±48.9	995.7±61.1	1260.9±77.3
Male		1049.4±38.8				1112.8±87.9	987.0±62.6	1277.2±80.8
		1108.7±77.2				999.7±48.9	1073.9±43.0	1181.3±235.8
CO (L·min ⁻¹)								
Female	N.A.	4.1±0.1	N.A.	N.A.	N.A.	4.2±0.1	4.0±0.1	4.2±0.2
Male		4.1±0.1				4.2±0.1	3.9±0.1	4.2±0.2
		4.2±0.2				3.9±0.3	4.3±0.3	4.7±0.7

Data are expressed as means ± S.D. N.A., data not available.

The blood samples (for DNA extraction) from IPAH patients were obtained from IPAH patients underwent right heart catheterization. The normal subjects (non-smokers) had no history of cardiopulmonary disease, drug abuse, and HIV/AIDS. The blood samples from normal subjects were collected by phlebotomists, while the blood samples from IPAH patients were collected during right heart catheterization via the catheter.

Identification of SNP in TRPC6 gene promoter region. To identify SNPs in the 5'- promoter region of *TRPC6*, approximately a 2000-bp size of *TRPC6* 5'-regulatory region sequence and total *TRPC6* gene sequence were obtained from the human chromosome 11 clone: RP11-223E3 (AP003080) in human genomic database. Six paired amplification PCR primers (Supplementary

Table 2) were designed to amplify six overlapping DNA segments spanning 2000 bp upstream and 110 bp downstream of the transcriptional start site of human *TRPC6*.

PCR amplification of genomic DNA encoding the putative *TRPC6* promoter region was performed by a GeneAmp PCR System (Perkin Elmer) using a Platinum PCR Supermix Kit (Invitrogen). The PCR products were detected by agarose gel electrophoresis, purified with a Gel extraction kit (Qiagen), and sequenced. The sequencing data were analyzed by Chromas software and compared with known SNPs deposited in the NCBI SNP databank. Vertebrate transcription factor binding sites within the 5'-regulatory region of human *TRPC6* were identified by Dragon TF association miner (<http://research.i2r.a-star.edu.sg/DRAGON/TFAM/v1.html>).

Supplementary Table 2. PCR Primers used for amplifying six overlapping DNA segments of the human *TRPC6* gene

Amplicon	Size (bp)	Sense/Antisense	Location (nt.)*
1	466	5'-TTCCCCATACTTGGTGTGCC-3'/ 5'-AGCAGAAACCACTGTGTCTG-3'	-2040 to -2020 -1595 to -1575
2	491	5'-CCTGTGTCAAATACGAAGCTGG-3'/ 5'-GGAGGCAAAGAAGCAGATGAG-3'	-1622 to -1601 -1152 to -1132
3	426	5'-AATTGGTCCCCCTTTCTGCCAC-3'/ 5'-AACGCCAGATGTTCCCAGTTCC-3'	-1201 to -1180 -797 to -776
4	451	5'-TTGCATTCGCAGTGACGGAAGG-3'/ 5'-AGAAAGCAGCCAAAGCCTGTCC-3'	-905 to -926 -476 to -497
5	490	5'-AGAAAGAAGAGGCTCGTGTCC-3'/ 5'-GAAAAGTCACCACTTAAGGGGG-3'	-676 to -656 -208 to -229
6	439	5'-TTGCTCTCCGCTCTTACGCTTC-3'/ 5'-TCCATGAGCAGATAGTCCTGGC-3'	-335 to -356 +83 to +104

* Nucleotide locations related to the 5'-upstream of the translational start site (ATG). The fidelity and specificity of the primer oligonucleotides were examined using the BLAST program.

Supplementary Table 3. Allele frequency of the *TRPC6* promoter SNPs sorted by cohort

Position (nt.)	SNP	Allele	Normal (n=237)		IPAH (n=268)		Odds Ratio (95% CI)		P value	
			USA	DE	USA *	DE	USA	DE	USA	DE
-316	A→T	A	197 (77.6%)	157 (71.4%)	284 (77.2%)	118 (70.3)	1.02 (0.69-1.49)	1.06 (0.68-1.64)	0.91	0.81
		T	57 (22.4%)	63 (28.6%)	84 (22.8%)	50 (29.7%)				
-254	C→G	C	235 (92.5%)	209 (95.0%)	319 (86.7%)	154 (91.7%)	1.89 (1.09-3.31)	1.73 (0.76-3.91)	0.02	0.19
		G	19 (7.5%)	11 (5.0%)	49 (13.3%)	14 (8.3%)				
-218	C→T	C	197 (77.6%)	157 (71.4%)	284 (77.2%)	118 (70.3)	1.02 (0.69-1.49)	1.06 (0.68-1.64)	0.91	0.81
		T	57 (22.4%)	63 (28.6%)	84 (22.8%)	50 (29.7%)				

* Only IPAH samples were obtained from Vanderbilt. Totals shown here reflect combined UCSD and Vanderbilt IPAH cohorts so that proper comparisons may be made between the different cohort

normal and IPAH groups. As stated in the main text, there was no significant difference between mean blood pressure in NPH and IPAH subjects. In IPAH patients alone, there was no significant difference of mean blood pressure among patients with the wild-type -254C/C (88.7±15.15 (SD) mmHg, n=195), and the mutated -254C/G (86.0±13.47 (SD) mmHg, n=29, P=0.36308) and -254G/G (86.6±9.16 mmHg, n=8, P=0.69518) alleles. Furthermore, the blood pressure in IPAH patients with the G allele (-254C/G and -254G/G combined; 89.8±12.17 (SD) mmHg, n=14) was the same as that in normal subjects with the G allele (-254C/G and -254G/G combined; 86.2±12.55 (SD) mmHg, n=37; P=0.35825).

Preparation of cytoplasmic and nuclear extracts from PSMCs. Cytosolic and nuclear extracts from cultured PSMCs were collected using a modified protocol. Briefly, cells were washed with PBS and incubated in cytoplasmic extraction buffer [10 mM Tris-HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, 0.4% NP-40, 1mM DTT and the protease inhibitor cocktail (Complete Mini, Roche Diagnostics)] for 10 min on ice, the samples were then scraped and collected. After centrifuged at 1000× g for 5 min, extracted soluble material representing the cytoplasmic fraction was isolated. The pellet was further extracted in the nuclear extraction buffer [50 mM Tris-HCl (pH 8.0), 410 mM NaCl, 1.5 mM MgCl₂, 25% glycerol] for 10 min on ice to isolate the nuclear fraction. Extracted soluble material representing the nuclear fraction was isolated after centrifuged at 16000× g for 10 min at 4°C. The paired cytoplasmic and nuclear fractions from PSMCs were stored at -20°C before use.

RNA isolation and RT-PCR. Total RNA was isolated from PSMC using TRIzol reagent (Invitrogen). Two µg of RNA was used to synthesize first-strand cDNA using the Super-Script[®] First-Strand Synthesis System (Invitrogen). PCR was performed by a GeneAmp PCR System (Perkin-Elmer) using the Platinum PCR Supermix (Invitrogen). The sense and antisense primers are specifically designed from the coding regions of *TRPC* genes (*TRPC4* and *TRPC6*) and GAPDH (Supplementary Table 4).

Supplementary Table 4. Oligonucleotide sequences of primers used for RT-PCR*

Genes	Predicted Size (bp)	Sense/Antisense	Location (nt.)
TRPC4 (NM_016179)*	415	5'-TCTGCAAATATCTCTGGGAAGAATGC-3'/ 5'-AGAAAGCAGCCAAAGCCTGTCC-3'	1743 to 1768 2131 to 2157
TRPC6 (NM_004621)*	326	5'-AGTCCGGCTTACCTGTCATTGT-3'/ 5'-ATGGAGAGAAGTTGCTGTTGGC-3'	1271 to 1292 1575 to 1596
GAPDH (NM_002046)*	550	5'-CTTTGGTATCGTGGAAGGACTC-3'/ 5'-TCTTCCTCTGTGCTCTTGCTG-3'	603 to 624 1131 to 1152

* Accession numbers in GenBank for the sequence used in designing the primers.

Western blot analysis. The cytoplasmic extract (10 µg of protein), the nuclear extract (20 µg of protein) or total cell lysates (30-50 µg of protein) were electrophoresed on NuPAGE[®] 4-12% Bis-Tris gels (Invitrogen) and transferred to a nitrocellulose membrane. Polyclonal antibodies against p50, p65 (Upstate), IκBα (Cell Signaling) and TRPC6 (Chemicon); monoclonal antibodies against p84, histone H2, and GAPDH were used as primary antibodies. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as secondary antibodies. The bound antibody was detected using an enhanced chemiluminescence detection system (Amersham).

Immunofluorescence staining. PSMCs on cover slips were fixed in 4% paraformaldehyde/PBS, permeabilized and exposed to rabbit anti-TRPC6 antibody (Chemicon). FITC-conjugated goat anti-rabbit IgG was used to detect TRPC6. HA epitope tag was stained by TRITC-conjugated anti-HA mouse antibody. The cells on cover slips were visualized using a confocal laser scanning microscope (LSM510 META, Zeiss).

Generation of recombinant adenovirus carrying human *IκBαSR*. The *IκBα*-super repressor (mutant *IκBα*, *IκBSR*) cDNA (S32A/S36A) was cloned into the pShuttle by digesting the pCMV-*IκBαM* with BamHI/HindIII and ligating the resulting fragments into the XbaI site of the pShuttle vector. The pAdeno-X/*IκBSR* (Ad-*IκB*) was constructed by digesting pShuttle constructs with PI-SceI/I-CeuI and ligating the resulting fragments into the PI-SceI/I-CeuI sites of the pAdeno-X adenoviral vector. Recombinant adenoviral plasmids were packaged into infectious adenoviral particles by transfecting HEK-293 cells using LipofectAMINE PLUS reagent. The adenoviral particles were propagated in HEK-293 cells and purified on CsCl ultracentrifugation. Titers of the adenoviral stock were determined by standard plaque assay. Recombinant adenoviruses were screened for expression of the introduced gene by Western blot. pAdeno-X, which was the recombinant replication-incompetent adenovirus carrying no *IκBSR* cDNA insert, was used as a control adenovirus (Ad-null).

Generation of recombinant adenovirus carrying human *TRPC6* specific siRNA. Recombinant adenovirus carrying siRNA targeting on human *TRPC6* was generated using an Invitrogen Expression Kit. We also constructed DNA-based vector (*pSilencer 2.0-U6-TRPC6*) that expressed *TRPC6* siRNA targeting on AAGGTCTTTATGCAATTGCTG (nucleotide 2214-2234 of *TRPC6* mRNA, NM_004621). The siRNA expression DNA cassettes, including RNA Polymerase III promoter U6, a hairpin siRNA target sequence and terminator, were subcloned into pENTR1A entry vector (Invitrogen). A scrambled control vector and GAPDH specific siRNA vector were also constructed. After efficient recombination of the entry vector into the promoterless pAd/PL-DEST Gateway® vectors, followed by viral production and transduction. The adenoviral particles for *TRPC6*-siRNA, GAPDH-siRNA and scramble control were plaque-purified and amplified in 293A cells. The titer of the adenoviral stock was measured by spectrophotometer. For adenovirus infection experiments, cells were infected with the appropriate virus in SMBM containing 0.2% FBS for 4 hrs and used for experiments 24 to 48 hours after adenoviral infection.

Cytosolic $[Ca^{2+}]$ measurement. Cells were loaded with fura-2/AM (3 μ M) in culture media for 30 min, then mounted in a perfusion chamber on the stage of a Zeiss Axiovert 35 microscope equipped with a 40 \times plan-Neofluar objective (NA 1.30 oil). Cells were then washed for 40 min by Ringer's solution to remove extracellular dye and allow intracellular esterases to cleave cytosolic fura 2-AM into active fura 2. Alternating 340 or 380 nm light was controlled by a wave length switcher (Lambda DG-4, Sutter Instruments) to elicit fura-2 fluorescence. A 400 nm dichroic mirror and 480 nm long-pass emission filter (Chroma) supplied light to a Photometrics CoolSNAP HQ CCD camera (Roper Scientific). Images were acquired with Metafluor software (Universal Imaging Corp.). $[Ca^{2+}]_{cyt}$ was calculated from fura-2 fluorescent emission excited at 340 and 380 nm (F_{340}/F_{380}) using the ratio method based on the following equation: $[Ca^{2+}]_{cyt} = K_d \times (Sf_2/Sb_2) \times (R - R_{min})/(R_{max} - R)$, where K_d (225 nM) is the dissociation constant

for Ca^{2+} , Sf_2 and Sb_2 are emission fluorescence values at 380-nm excitation, R is the measured fluorescence ratio, and R_{\min} and R_{\max} are minimal and maximal ratios, respectively. The $[\text{Ca}^{2+}]_{\text{cyt}}$ in each condition was determined as the average from 10 to 20 cells, and experiments were repeated at least four times.

Electrophysiological measurements. PASMCs were cultured on cover slips and mounted in a perfusable recording chamber on a Zeiss inverted microscope immediately before use. Membrane currents and capacitance were recorded with an EPC-9 amplifier coupled to ITC16 interface using whole-cell patch-clamp technique. Patch pipettes (2-2.5 M Ω) were pulled on a Sutter electrode puller using borosilicate glass capillary tubes, and fire polished on a Narishige microforge. Na^+ -containing Ringer solution was used as the extracellular (bath) solution and contained (in mM): NaCl 160, KCl 4, CaCl_2 2, MgCl_2 1, and HEPES 10 (pH 7.4). The pipette (intracellular) solution contained (in mM): cesium aspartate (CsAsp) 145, MgCl_2 2, CaCl_2 0.3, EGTA 10, and HPES 10 (pH 7.2 and pCa 8). Voltage ramps ranging from -100 mV to +100 mV were applied from a holding potential of 0 mV (which inactivates voltage-gated Na^+ and Ca^{2+} channels). All experiments were performed at room temperature (24°C). The TRPC6 currents were activated by application of 100 μM OAG for 10-15 min.

The -254(G-to-C) SNP in normal subjects and IPAH patients in African Americans. In addition to Caucasian patients, the -254(C→G) SNP in the *TRPC6* gene was also found in two African American IPAH patients; the frequency of the -254G allele in African American patients is also significantly higher than in African American controls. As shown in Supplementary Table 5, the allele frequency of the -254(C→G) SNP is 18.8% in African American IPAH patients (n=8; vs. 1.3% in African American normal controls, n=38). One of the two African American IPAH patients is a homozygote (-254G/G), and the other is a heterozygote (-254C/G). These data imply that the difference is not limited in Caucasians.

Supplement Table 5. The -254G allele frequency is significantly higher than in IPAH patients than in normal subjects in African Americans

Genotype Frequency					Allele Frequency			
	Normal (n=38)	IPAH (n=8)	Odds Ratio	P- value	Normal (n=38)	IPAH (n=8)	Odds Ratio (95% CI)	P- value
-254 C→G					C allele	C allele		
-254 C/C	37 (89.3%)	6 (75.0%)			75 (98.7%)	13 (81.2%)		
-254 C/G	1 (2.6%)	1 (12.5%)			G allele	G allele		
-254 G/G	0 (0.9%)	1 (12.5%)	12.3 ^b (0.96~158)	0.07	1 (1.3%)	3 (18.8%)	17.3 (1.7~179)	<0.02
-361 A→T					A allele	A allele		
-361 A/A	29 (76.3%)	7 (87.5%)			67 (88.2%)	15 (93.7%)		
-361 A/T	9 (23.7%)	1 (12.5%)			T allele	T allele		
-361 T/T	0(0%)	0 (0%)	0.59 ^a (0.06-5.63)	0.55	9 (11.8%)	1 (6.3%)	0.50 (0.06-4.22)	0.51

P -values, odds ratio and 95% confidence interval (95% CI) are calculated by χ^2 analysis for comparison of the genotype frequencies and allele frequencies between IPAH patients and normal subjects in African American. ^a, odds ratio and P values for (-361 A/T and -361 T/T) versus -361A/A and for (-218 C/T and -218 T/T) versus -218C/C; ^b, odds ratio and P value for

(-254 C/G and -254 G/G) versus -254 C/C. The -361(A→T) and -218(C→T) SNPs are in complete linkage disequilibrium. Since their allele frequencies, odds ratio, and *P* values are identical, only the -361A→T SNP is shown for the sake of clarity.

Differential regulation of NF-κB expression in -254C/G and -254C/C IPAH patient PASM C.

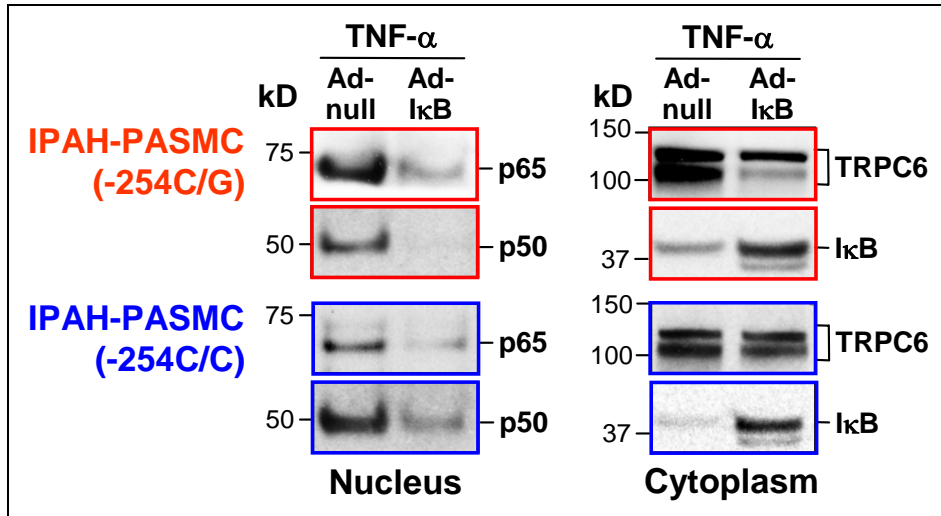
We have isolated PASM C from 4 IPAH patients, three of whom have a -254C/G genotype and one has the -254C/C genotype. Despite strenuous efforts, we have been unable to get tissue from a -254G/G allele patient prior to publication of the current study. Patient demographics and hemodynamics are reported in Supplement Table 6. Our observations imply that TRPC6 is upregulated in PASM C from all IPAH patients, however, NF-κB only upregulates TRPC6 expression in PASM C from patients who have a -254G allele.

Using PASM C from -254C/G and -254C/C individuals, we examined the regulation of TRPC6 expression by TNF-α. In the presence of TNF-α, the protein expression level of TRPC6 was not significantly different in the -254C/C IPAH PASM C infected with control adenovirus (Ad-null) and IκBα super-repressor (Ad-IκB), a nondegradable mutant dominant-negative IκBα variant that inhibits NF-κB activation and/or nuclear translocation of p65 and p50 (Supplementary Figure 1). However, in the -254C/G IPAH PASM C, the TRPC6 protein level was significantly reduced by IκBα super-repressor (Ad-IκB). These results suggest that NF-κB-mediated TRPC6 upregulation only occurs in PASM C from -254G allele patients.

Supplement Table 6. Demographics and hemodynamics of -254G and -254C IPAH patients with isolated PASM C.

	-254C/G	-254C/G	-254C/G	-254C/C
Gender	Male	Female	Female	Female
Race	White	White	White	Asian
Age (yr)	32	57	48	16
Mean PAP (mmHg)	53	66	51	65
PVR (dyn·min ⁻¹ ·cm ⁻⁵)	1450	1200	1533	1267
CO (L·min ⁻¹)	2.90	4.90	2.40	3.6
-254 Genotype	C/G	C/G	C/C	C/G

Supplement Figure 1. The -254G allele regulates NF-κB-mediated protein TRPC6 upregulation.



Supplement Figure 1. The -254G allele regulates NF-κB-mediated protein TRPC6 upregulation. Representative gels depict data from nuclear (A) and cytoplasmic (B) protein fractions from TNF-treated PASMC isolated from heterozygous (-254C/G: top) and homozygous (-254C/C: bottom) IPAH patients. Both fractions were treated with (Ad-IκB) or without (Ad-null) IκBα super-repressor. **Left.** IκBα treatment inhibits significantly decreases nuclear translocation of the p65 and p50 in both -254C/G and -254C/C PASMC. **Right.** TRPC6 expression is evaluated in Ad-null and Ad-IκB treated cells. IκB expression is enhanced by Ad-IκB in both -254C/G and -254C/C cell types. However, only the -254C/G IPAH cells exhibit decreased TRPC6 protein expression. Data for the -254C/G patient is reproduced from Figure 7.