c-Jun Decreases Voltage-Gated K⁺ Channel Activity in Pulmonary Artery Smooth Muscle Cells

Ying Yu, MD, PhD*; Oleksandr Platoshyn, MS*; Jifeng Zhang, PhD*; Stefanie Krick, MD; Ying Zhao, BS; Lewis J. Rubin, MD; Abraham Rothman, MD; Jason X.-J. Yuan, MD, PhD

**Background**—Activity of voltage-gated K⁺ (Kᵥ) channels controls membrane potential (Eₘ) that regulates cytosolic free Ca²⁺ concentration ([Ca²⁺]ₖᵢтен) by regulating voltage-dependent Ca²⁺ channel function. A rise in [Ca²⁺]ₖᵢтен in pulmonary artery smooth muscle cells (PASMCs) triggers vasoconstriction and stimulates PASMC proliferation. Whether c-Jun, a transcription factor that stimulates cell proliferation, affects Kᵥ channel activity in PASMCs was investigated.

**Methods and Results**—Infection of primary cultured PASMCs with an adenoviral vector expressing c-jun increased the protein level of c-Jun and reduced Kᵥ currents (I_Kᵥ) compared with control cells (infected with an empty adenovirus). Using single-cell reverse transcription–polymerase chain reaction, we observed that the mRNA level of Kv1.5 and the current density of I_Kᵥ were both attenuated in c-jun-infected PASMCs compared with control cells and cells infected with antisense c-jun. Overexpression of c-Jun also upregulated protein expression of Kvβ₂ and accelerated I_Kᵥ inactivation. Furthermore, Eₘ was more depolarized and [³H]thymidine incorporation was greater in PASMCs infected with c-jun than in control cells and cells infected with antisense c-jun.

**Conclusions**—These results suggest that c-Jun–mediated PASMC proliferation is associated with a decrease in I_Kᵥ. The resultant membrane depolarization increases [Ca²⁺]ₖᵢтен and enhances PASMC growth. (Circulation. 2001;104:1557-1563.)

**Key Words:** transcription factors  ■  ion channels  ■  genes  ■  lung  ■  remodeling

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**Pulmonary vascular remodeling due to proliferation and hypertrophy of pulmonary artery smooth muscle cells (PASMCs) is an important pathological feature in pulmonary hypertension.**¹⁻² A rise in cytosolic free Ca²⁺ concentration ([Ca²⁺]ₖᵢтен) is a major trigger for pulmonary vasoconstriction and an important stimulus for PASMC growth.³⁻⁵ [Ca²⁺]ₖᵢтен is increased primarily by Ca²⁺ release from intracellular Ca²⁺ stores and Ca²⁺ influx through Ca²⁺ channels in the plasma membrane.⁶⁻⁸ Among various Ca²⁺-permeable channels, the voltage-dependent Ca²⁺ channels (VDCCs) that are opened by membrane depolarization are a major Ca²⁺ entry pathway in vascular smooth muscle cells.⁹⁻¹⁰

Membrane potential (Eₘᵢ₀) is controlled primarily by the activity of Na⁺,K⁺-ATPase and the permeability of K⁺ ions across the plasma membrane through K⁺ channels. When K⁺ channels close or K⁺ channel expression is downregulated, whole-cell K⁺ currents decline and Eₘᵢ₀ becomes less negative.¹¹ The membrane depolarization opens VDCCs, promotes Ca²⁺ influx, increases [Ca²⁺]ₖᵢтен,¹²⁻¹⁵ and stimulates PASMC growth.¹²,¹³ Membrane depolarization may also promote Ca²⁺ entry via the reverse mode of Na⁺/Ca²⁺ exchange, which is sufficient to trigger Ca²⁺ release from ryanodine-sensitive Ca²⁺ stores, and increase [Ca²⁺]ₖᵢтен.¹⁴ In vascular smooth muscle cells, voltage-gated K⁺ (Kᵥ) channels play an important role in the regulation of resting Eₘᵢ₀.¹⁰⁻¹³ Blockade of Kᵥ channels causes membrane depolarization, opens VDCCs, induces Ca²⁺-dependent action potentials, and increases [Ca²⁺]ₖᵢтен in PASMCs.¹⁰⁻¹³

**c-jun** is an immediate-early gene whose mRNA expression increases rapidly and transiently when quiescent cells are stimulated to grow.¹⁵ c-Jun is a nuclear protein that serves as a nuclear intermediate of signal transduction in cellular growth and differentiation.¹⁶ How c-Jun mediates PASMC growth is unclear. This study was designed to test the hypothesis that c-Jun induces PASMC proliferation and hypertrophy partially by regulating expression and function of Kᵥ channels. The subsequent decrease in whole-cell Kᵥ currents (I_Kᵥ) induces membrane depolarization, increases [Ca²⁺]ₖᵢтен, and stimulates PASMC proliferation.

**Methods**

**Cell Preparation**

Primary cultured PASMCs were prepared from Sprague-Dawley rats as previously described.⁶,¹₀,¹² Briefly, adventitia and endothelium were carefully removed from the isolated pulmonary arterial branches (third to fourth division). The smooth muscle was digested...
Oligonucleotide Sequences of the Primers Used for Single-Cell RT-PCR

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<tr>
<th>Standard Names</th>
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*The accession numbers in GenBank for the sequences used in designing the primers.

with collagenase and elastase. The cells were plated onto coverslips or in flasks and cultured in 10% FBS-DMEM in a 37°C, 5% CO₂, humidified incubator.

Generation of Repombinant Adenoviral Vector and c-Jun Infection Protocol

E1 region–deleted recombinant adenoviral vectors carrying either sense (+ c-Jun) or antisense (− c-Jun) cDNA were constructed. A 2.6-kb-pair fragment of full-length c-Jun cDNA was then subcloned in sense or antisense orientation into the pACCMVpLpa shuttle vector to yield the sense and antisense constructs, pSR−sense-c-Jun and pSR−antisense-c-Jun. Both pSR−sense-c-Jun and pSR−antisense-c-Jun were then independently co-transfected with pJM17 into HEK-293 cells by calcium phosphate/DNA coprecipitation. For viral plaque assays, the cotransfected HEK-293 cells were overlaid with 0.65% agarose (prepared with 1% milk powder), the membranes were incubated with rabbit anti–c-Jun and anti-Kv β polyclonal antibodies (Biosource). The membranes were then washed and incubated with anti-mouse horseradish peroxidase–conjugated IgG for 90 minutes at 24°C. The bound antibody was detected with an enhanced chemiluminescence detection system (Amersham).

Single-Cell RT-PCR

Multiplex single-cell reverse transcription (RT)-PCR was performed to determine the mRNA expression of c-Jun and Kv1.5 at the single-cell level. After I_{V(K)} had been recorded, the cell was carefully aspirated into a collection pipette that contained 12 µL of the pipette solution supplemented with 10 µM TTH polymerase. The content in the pipette was then expelled immediately into a 0.2-mL PCR tube that contained 8 µL of the solution composed of (mmol/L) Tris-HCl 10, KC1 50, MgCl₂ 2.5, diethiothreitol 0.1, oligo(dT) 1.25, and dNTPs 0.5, and 5 U AMV reverse transcriptaseXL. RT was performed for 60 minutes at 42°C. Then, first-round PCR with 45 cycles was performed in the same tube by the addition of 80 µL of the premixed PCR buffer containing 10 mmol/L Tris-Cl 50, 2.5 mmol/L MgcL₂ 20 mmol/L each of sense and antisense primers (first primers) for all the genes of interest, and 5 U Taq polymerase (RNA PCR kit, Takara). Two-microliter aliquots of the first-round PCR products were reamplified by the second-round PCR with 25 to 30 cycles, which was carried out separately with fully nested gene-specific primers (nested primers) for each target gene. The second-round PCR amplification products were separated on 1.5% agarose gel and visualized with GelStar gel staining. The cell-free samples were also used in PCR as a negative control. To semiquantify the PCR products, an invariant mRNA of β-actin was used as an internal control. The sense and antisense primers were specifically designed from the coding regions of rat c-Jun (X17215) and Kv1.5 (M27158) (Table).

Determination of DNA Synthesis

DNA synthesis was evaluated by [³H]thymidine incorporation. Cells were first cultured in serum-free DMEM for 24 hours and then infected with the adenoviral vector carrying + c-Jun or − c-Jun for 3 hours in 0.2% FBS-DMEM. [³H]thymidine (1 µCi/well) was added after 48 hours, and the incorporated radioactivity was determined by a liquid scintillation counter 12 hours later. The results are repre-
sented as mean counts per minute from 9 to 12 experiments. For 4-AP experiments, the cells were incubated for 20 minutes, 3 times intermittently during a period of 24 hours, in 0.2% FBS-DMEM containing 1.25 mmol/L 4-AP before [3 H]thymidine was added.

Statistical Analysis
The composite data are expressed as mean±SEM. Statistical analyses were performed by use of unpaired Student’s t test or 1-way ANOVA and Fisher’s protected least significant difference (PLSD) tests where appropriate. Differences were considered to be significant at a value of *P*<0.05.

Results

Overexpression of c-Jun Decreases *I*<sub>K(V)</sub> in PASMCs

The protein level of c-Jun was significantly higher in rat PASMCs infected with the adenovirus expressing +c-jun than in cells infected with control adenovirus that does not carry the c-jun gene (Cont) and cells infected with +c-jun or A-c-jun (Figure 1A). Overexpression of c-Jun was associated with a significant decrease in amplitude of *I*<sub>K(V)</sub> (Figure 1B). The averaged current amplitudes at +40 mV were 25±4 pA in control cells and 12±2 pA (*P*<0.01) in the c-jun-infected cells (Figure 1C). Infection of c-jun negligibly affected membrane capacitance (*C<sub>m</sub>*) (Figure 2A) but markedly reduced current density (Figure 2C) of *I*<sub>K(V)</sub>

The relationships of current-density and voltage show that overexpression of c-Jun decreased the current-density of *I*<sub>K(V)</sub> by ≈62% at +80 mV (from 56.6±7.3 to 21.3±1.8 pA/pF) (Figure 2C, inset).

mRNA Level of c-Jun Is Inversely Proportional to the Amplitude of *I*<sub>K(V)</sub> in Single PASMCs

The level of c-Jun mRNA was much higher (a) and the amplitude of whole-cell *I*<sub>K(V)</sub> (b and c) was markedly lower in a c-jun-infected cell than in a control cell (Figure 3A). Furthermore, the level of c-Jun mRNA was much lower and the amplitude of *I*<sub>K(V)</sub> was much higher in a cell infected with antisense c-jun than in a cell infected with c-jun (Figure 3B). The same results were reproduced in 5 pairs of control and c-jun-infected cells.

Overexpression of c-Jun Downregulates the mRNA Expression of the K<sub>v</sub> Channel α-Subunit

After recording of *I*<sub>K(V)</sub>, the PASMC was collected to determine mRNA expression of Kv1.5 by RT-PCR. As shown in Figure 4, the Kv1.5 mRNA level in a cell infected with c-jun was much lower than in a control cell, whereas the β-actin mRNA level was similar (Figure 4A). Furthermore, in the c-jun–infected cell, the decreased mRNA expression of...
Kv1.5 correlated with the diminished amplitude of whole-cell\(I(V)\) (Figure 4B). The same results were reproduced in 5 pairs of the cells. These results suggest that c-Jun may decrease \(I(V)\) by affecting both the function and expression of Kv channels in PASMCs.

Overexpression of c-Jun Stimulates Kv\(\beta_2\) Protein Expression and Accelerates \(I(V)\) Inactivation

The Kv channel \(\beta\)-subunit is a regulatory subunit that confers inactivation on the Kv channel \(\alpha\)-subunits (eg, Kv1.5) and blocks Kv channels as an open-channel blocker.\(^{19-21}\) Therefore, an increase in \(\beta\)-subunit expression should decrease \(I(V)\).

In cells infected with \(c-jun\), the current inactivation was accelerated, whereas the current activation appeared to be unaffected, compared with control cells. The time constants for the current inactivation (\(\tau_{inact}\)) at +80 mV were 247±34 and 125±36 ms (\(P<0.01\)) in controls and \(c-jun\)-infected cells, respectively (Figure 5A to 5C). Furthermore, the protein level of Kv\(\beta_2\) was significantly greater in the \(c-jun\)-infected cells than in control cells (Figure 5D), suggesting that c-Jun upregulates protein expression of the Kv channel \(\beta\)-subunit.

Overexpression of c-Jun and Blockade of Kv Channels Cause Membrane Depolarization and Stimulate PASMC Proliferation

To test the effects of c-Jun on resting \(E_m\) and cell proliferation, we compared \(E_m\) and thymidine incorporation in...
PASMCs infected with an empty adenovirus (Cont) and with adenoviral vectors carrying +c-jun and A-c-jun. As shown in Figure 6, [3H]thymidine incorporation was markedly increased (similar results were reproduced 3 times in cells isolated from 3 rats), and resting Em was much depolarized in cells infected with +c-jun, compared with control cells and cells infected with A-c-jun. Furthermore, pharmacological blockade of K_v channels with 4-AP (1.25 mmol/L) caused E_m depolarization and significantly increased [3H]thymidine incorporation in control cells (similar results were reproduced 3 times in cells isolated from 3 rats). These results suggest that the c-Jun–induced decrease in I_{K(V)} stimulates DNA synthesis in PASMCs by causing membrane depolarization and increase in [Ca^{2+}]_{cyt}.

Discussion

Subsequent to the activation of immediate-early genes (eg, c-jun), the cellular signaling pathways that cause proliferation and hypertrophy of PASMCs are not well understood. In primary cultured rat PASMCs, we observed that overexpression of c-Jun reduced I_{K(V)} by downregulating Kv1.5 expression and upregulating Kvβ2 expression and induced membrane depolarization. It has been shown that in PASMCs, opening of VDCCs by membrane depolarization causes increased [Ca^{2+}]_{cyt} and induces cell contraction and proliferation. In this study, the c-Jun–mediated decreases in I_{K(V)} and membrane depolarization were also associated with an increase in PASMC proliferation. These observations suggest that c-Jun–mediated PASMC growth may result from the regulation of K_v channel expression and function, the activity of K_v channels serving as an effector to prompt cell proliferation by modulating E_m and [Ca^{2+}]_{cyt}.

An increase in c-Jun mRNA level was associated with thrombin-induced hypertrophy and PDGF-mediated proliferation of rat PASMCs. c-Jun is a transcription activator that

Figure 5. c-Jun accelerates I_{K(V)} inactivation and upregulates Kvβ2 expression. A, Currents (at +80 mV) averaged from 53 control cells (Cont) and 63 cells infected with (+c-jun). Normalized average currents (at +80 mV) showing current inactivation (B) and summarized data (mean±SEM) showing time constants (C) for current activation (τ_{act}) and inactivation (τ_{inact}) in control cells and cells infected with +c-jun. D, Western blot analysis of Kvβ2 protein and summarized data (mean±SEM) showing protein levels of Kvβ2 (normalized to level of α-actin) in control and c-jun–infected cells. **P<0.01 vs Cont.

Figure 6. Effects of c-Jun and 4-AP on E_m and PASMC proliferation. A, Summarized data (mean±SEM) showing [3H]thymidine incorporation (n=6 cell samples for each group) and resting E_m (n=16 cells from 6 rats) in control cells (Cont) and cells infected with +c-jun or A-c-jun. B, Summarized data (mean±SEM) showing [3H]thymidine incorporation (n=12 cell samples for each group) and resting E_m (n=20 cells from 3 rats) in control cells treated with 4-AP (4-AP) or without (Cont) 4-AP (1.25 mmol/L). ***P<0.001 vs Cont.
upregulates responsive genes associated with proliferation, differentiation, and apoptosis.\textsuperscript{16,23} c-Jun may indirectly regulate transcription of Kv1.5 gene by activating expression of an intermediate gene product that can subsequently down-regulate K\textsubscript{a} channel expression and decrease I\textsubscript{K(V)\textsubscript{a}}. The augmenting effect of c-Jun on Kv\textsubscript{a} expression implies that c-Jun modulates K\textsubscript{a} channel \(\alpha\)- and \(\beta\)-subunit gene expression by different mechanisms.

Native K\textsubscript{a} channels are homomeric or heteromeric tetramers that are composed of 2 structurally distinct subunits: the pore-forming \(\alpha\)-subunits and the regulatory \(\beta\)-subunits. Kv1.5 is a delayed rectifier K\textsubscript{a} channel \(\alpha\)-subunit that has been described in PASMCs from animals and humans.\textsuperscript{24} The homomeric Kv1.5 channels, which are activated at potentials ranging from \(-30\) to \(-60\) mV, appear to be responsible for regulating resting E\textsubscript{m}, whereas the heteromeric Kv1.5/Kv1.2 channels are involved in mediating membrane depolarization during hypoxia in PASMCs.\textsuperscript{25} The K\textsubscript{a} channel \(\beta\)-subunit has been demonstrated to block the K\textsubscript{a} channel \(\alpha\)-subunits as an open-channel blocker,\textsuperscript{21} confer fast inactivation on delayed rectifier K\textsubscript{a} channel \(\alpha\)-subunits,\textsuperscript{19,20} and confer oxygen and redox sensitivity on K\textsubscript{a} channel \(\alpha\)-subunits.\textsuperscript{20,25} Therefore, the c-Jun-mediated decrease in I\textsubscript{K(V)} expression and increase in Kv\textsubscript{b}\textsubscript{2} expression would reduce the number of homomeric Kv1.5 channels and increase the number of heteromeric Kv1.5/Kv1.2 channels, thus decreasing the amplitude and current density of I\textsubscript{K(V)}.

E\textsubscript{m} in PASMCs is regulated primarily by K\textsuperscript{\textsuperscript{+}} permeability, which is determined by sarcosomal K\textsuperscript{\textsuperscript{+}} channel activity.\textsuperscript{11} Under resting conditions, K\textsuperscript{\textsuperscript{+}} permeability through K\textsubscript{a} channels is partially responsible for determining E\textsubscript{m} in smooth muscle cells.\textsuperscript{10–13} Thus, E\textsubscript{m} is directly related to the whole-cell I\textsubscript{K(V)}: PASMCs have a very large membrane input resistance (1 to 10 G\textsubscript{\Omega})\textsuperscript{11}; therefore, a modest change in I\textsubscript{K(V)} would cause a large change in E\textsubscript{m}. Indeed, overexpression of c-Jun reduced I\textsubscript{K(V)} by 50% to 70% (at \(-40\) and \(+80\) mV) and caused a 15-mV depolarization. These results indicate that the c-Jun–mediated decrease in I\textsubscript{K(V)} is sufficient to cause substantial membrane depolarization in PASMCs.

Studies on the kinetics of L-type VDCCs and its relationship with [Ca\textsuperscript{2\textsuperscript{+}}]\textsubscript{cyt} have demonstrated that prolonged membrane depolarization at a range of \(-35\) to \(-20\) mV (a voltage range at which the Ca\textsuperscript{2\textsuperscript{+}} channel inactivation is incomplete while the channel activation begins) can open Ca\textsuperscript{2\textsuperscript{+}} channels sufficiently to cause a sustained increase in [Ca\textsuperscript{2\textsuperscript{+}}]\textsubscript{cyt}.\textsuperscript{15} Because of the minimal resistance of the nuclear membrane to Ca\textsuperscript{2\textsuperscript{+}} ions,\textsuperscript{26} the sustained elevation of [Ca\textsuperscript{2\textsuperscript{+}}]\textsubscript{cyt} would rapidly increase nuclear [Ca\textsuperscript{2\textsuperscript{+}}]. Furthermore, a very small increase in [Ca\textsuperscript{2\textsuperscript{+}}]\textsubscript{cyt} would also result in a large increase in [Ca\textsuperscript{2\textsuperscript{+}}]\textsubscript{cyt} in the sarcoplasmic reticulum, a cytoplasmic organelle involved in protein processing and lipid synthesis.\textsuperscript{6} In the cell cycle, Ca\textsuperscript{2\textsuperscript{+}} is necessary for transitions from the resting state (G\textsubscript{0}) to DNA synthesis and mitosis.\textsuperscript{4} Thus, increases in cytosolic, nuclear, and sarcoplasmic reticulum [Ca\textsuperscript{2\textsuperscript{+}}] all may contribute to stimulate PASMCs.

It has been demonstrated that an increase in [Ca\textsuperscript{2\textsuperscript{+}}]\textsubscript{cyt} due to Ca\textsuperscript{2\textsuperscript{+}} influx through VDCCs spatially stimulates transcription of c\texttextit{-fos}/c\texttextit{-jun} by activation of cAMP response element binding protein in the cytosol and nucleus.\textsuperscript{27,28} Overexpression of c-Jun decreased I\textsubscript{K(V)} and depolarized PASMCs. The resultant increase in [Ca\textsuperscript{2\textsuperscript{+}}]\textsubscript{cyt} due to opening of VDCCs should further stimulate c\texttextit{-fos}/c\texttextit{-jun} transcription. Therefore, this may serve as a positive-feedback mechanism in regulating Ca\textsuperscript{2\textsuperscript{+}}-sensitive genes, which are required for cell proliferation and hypertrophy.

In summary, the results from this study demonstrate that overexpression of c-Jun downregulates expression of the K\textsubscript{a} channel \(\alpha\)-subunit (Kv1.5) and upregulates expression of the \(\beta\)-subunit (Kv\textsubscript{b}\textsubscript{2}) in PASMCs. The resultant decrease in I\textsubscript{K(V)} causes membrane depolarization and stimulates cell proliferation by raising [Ca\textsuperscript{2\textsuperscript{+}}]\textsubscript{cyt}. A similar increase in [Ca\textsuperscript{2\textsuperscript{+}}]\textsubscript{cyt} and membrane depolarization due to decreased K\textsubscript{a} channel activity in PASMCs have been implicated in hypoxic pulmonary vasoconstriction\textsuperscript{29–31} and primary pulmonary hypertension.\textsuperscript{32} Further studies are necessary to determine whether upregulated c\texttextit{-jun} transcription and increased c-Jun function are responsible for the inhibited expression of K\textsubscript{a} channels in patients with primary pulmonary hypertension and the downregulation of K\textsubscript{a} channel \(\alpha\)-subunits in chronic hypoxia.

Acknowledgments

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