ASIC1-mediated calcium entry stimulates NFATc3 nuclear translocation via PICK1 coupling in pulmonary arterial smooth muscle cells

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CHRONIC HYPOXIA-INDUCED PULMONARY hypertension (World Health Organization Group III) is associated with respiratory diseases such as chronic obstructive pulmonary disease, interstitial lung diseases, and sleep-related breathing disorders such as sleep apnea. Chronic hypoxia (CH) results in enhanced vasoconstriction and vascular arterial remodeling due in part to increases in pulmonary vascular intracellular Ca2+i concentration ([Ca2+i]). Acid-sensing ion channel-1 (ASIC1) is expressed in pulmonary artery smooth muscle cells (PASMC); contributes to enhanced store-operated Ca2+i entry (SOCE); and is an important constituent to the active vasoconstriction, vascular remodeling, increased pulmonary arterial pressure, and right ventricular hypertrophy associated with hypoxic pulmonary hypertension (21, 39). ASIC1 is a member of the degenerin/epithelial sodium channel (DEG/ENaC) family and is permeable to both Na+ and Ca2+ (51, 56).

CH additionally activates the Ca2+/calcineurin-dependent transcription factor known as nuclear factor of activated T cells isoform-3 (NFATc3), in mouse PASMC (8, 9). Previous research in our laboratory demonstrated that NFATc3 is required for CH-induced pulmonary arterial remodeling. This process involves an initial proliferation of PASMC (dedifferentiation) followed by differentiation (upregulation of differentiation marker soluble guanylyl cyclase α1) and hypertrophy of PASMC (upregulation of smooth muscle-α-actin) (2, 7, 9). NFATc3 belongs to a family of four transcription factors (NFATc1, NFATc2, NFATc3, and NFATc4) that share the property of Ca2+/calcineurin-dependent nuclear translocation (reviewed in Ref. 15). We have shown that elevated endothelin-1 (ET-1) levels in response to CH contribute to NFATc3 activation through binding to type A ET receptors (8, 9). ET-1 causes release of Ca2+i from intracellular stores and Ca2+i influx through L-type voltage-gated Ca2+i channels, store-operated channels, and receptor-operated channel (49, 50). Administration of the L-type voltage-gated Ca2+i channel inhibitor diltiazem attenuated but did not abolish CH-induced NFATc3 activation in isolated pulmonary arteries from NFAT-luciferase reporter mice (8). These data are consistent with reports of other Ca2+i entry pathways being involved in NFATc3 activation; namely, SOCE (18, 20, 42, 52, 54). The goal of this study was to determine whether ASIC1-mediated SOCE contributes to CH-induced NFATc3 activation in PASMC.

Most intracellular Ca2+i signaling events take place through the calcium-modulated protein (calmodulin). In response to elevated Ca2+i levels, calmodulin binds to and activates either calmodulin-dependent protein kinases or the serine/threonine protein phosphatase calcineurin. Under resting conditions, NFAT proteins are phosphorylated and reside in the cytoplasm. Upon Ca2+-calmodulin-induced activation, NFAT proteins are dephosphorylated by calcineurin, translocate to the nucleus, and become transcriptionally active (16). We have recently shown that calcineurin limits ASIC1-mediated SOCE through dephosphorylation of the channel in rat PASMC (14). Furthermore, this response is dependent on the PDZ-binding protein known as protein interacting with C kinase (PICK1), which has been shown to bind both ASIC1 (11, 17) and calcineurin B...
(19). These data are consistent with previous reports demonstrating calcineurin-dependent dephosphorylation and inactivation of ASICs in neuronal cells (4). Together, these studies provide evidence of a functional interaction between ASIC1, PICK1, and calcineurin. Although these studies exhibit regulation of ASIC1 by calcineurin, it is also possible that ASIC1-mediated Ca^{2+} influx activates calcineurin and thus NFATc3. Therefore, in the present study we tested the hypothesis that ASIC1 contributes to NFATc3 nuclear translocation in PASMC. Furthermore, we hypothesized that ASIC1-stimulated NFATc3 nuclear translocation is dependent on the PDZ-binding protein PICK1.

METHODS

Animals and CH Exposure Protocol

ASIC1-knockout (ASIC1^{−/−}) mice were kindly provided by Drs. M.J. Welsh and J.A. Wemmie [University of Iowa, Iowa City, IA (53)]. Mice were bred on a C57BL/6 background, and ASIC1 wild-type (ASIC1^{+/+}) and ASIC1^{−/−} male and female mice (~12 wk old) were used equally for each protocol. Disruption of ASIC1 was confirmed by PCR and agarose gel electrophoresis using a three-primer system to detect both wild-type and disrupted alleles (39).

Animals designated for exposure to CH were housed in a hypobaric chamber with barometric pressure maintained at ~380 mmHg for 1 wk [our laboratory had previously demonstrated NFATc3 nuclear accumulation was greatest following 7 day CH (9)]. Age-matched control mice were housed at ambient barometric pressure (~630 mmHg in Albuquerque, NM). All protocols used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico School of Medicine.

Determination of Vasoreactivity and [Ca^{2+}], in Small Pulmonary Arteries

To determine changes in pulmonary vasoreactivity, [Ca^{2+}], and SOCE, small intrapulmonary arteries were cannulated and pressurized for simultaneous dimensional and [Ca^{2+}] analysis as previously described (39). Briefly, mice were anesthetized with pentobarbital sodium (200 mg/kg ip). The left lung was removed, and small intrapulmonary arteries (fourth to fifth order) were dissected free, transferred to a vessel chamber (Living Systems), and secured to tapered glass pipettes with a single strand of silk ligature. After cannulation, the artery was pressurized with a servo-controlled peristaltic pump (Living Systems) to 12 mmHg. Any artery that failed to maintain pressure upon switching off of the servo-controller was discarded. The vessel chamber was superfused with HEPES-based physiological saline solution (PSS; in mM: 130 NaCl, 4 KC1, 1.2 MgSO_{4}, 4 NaHCO_{3}, 10 HEPES, 1.18 KH_{2}PO_{4}, 6 glucose, 3 EGTA; pH adjusted to 7.4 with NaOH) containing 50 μM diltiazem (Sigma-Aldrich) to prevent Ca^{2+} entry through L-type voltage-gated Ca^{2+} channels, and 10 μM cyclopiazonic acid (CPA; Calbiochem) to deplete intracellular Ca^{2+} stores and prevent Ca^{2+} reuptake through the sarcoplasmic reticulum Ca^{2+}-ATPase. The changes in [Ca^{2+}], were determined upon repletion of HEPES-based PSS containing 1.8 mM CaCl_{2} in the continued presence of diltiazem and CPA. Area under the curve (AUC) was calculated as previously described (44).

ET-1 vasoconstrictor reactivity. ET-1 vasoconstrictor reactivity was assessed by superfusion (5 ml/min at 37°C) of cumulative concentrations of ET-1 (10^{-10} to 10^{-8} M; Sigma-Aldrich) in isolated pulmonary arteries from ASIC1^{+/+} and ASIC1^{−/−} mice exposed to control and CH conditions.

Contribution of ASIC1 to Hypoxia-Induced NFATc3 Nuclear Import

Isolated lungs were fixed with 4% formaldehyde in PBS, cryoprotected with 30% sucrose in PBS, embedded in optimal cutting temperature (OCT) medium, and frozen. Cryostat sections (10 μm) were stained with primary rabbit polyclonal anti-NFATc3 (1:100, Santa Cruz Biotechnology) and goat anti-α-actin (1:200, Abcam) followed by donkey anti-rabbit DyLight 649 and anti-goat DyLight 549 (Jackson ImmunoResearch Laboratories or Pierce) as previously described (6, 9). Nuclei were stained using SYTOX green (1:10,000, Molecular Probes). Specificity of immune staining was confirmed by the absence of fluorescence in tissues incubated with primary or secondary antibodies alone. Images of small pulmonary arteries were collected using a 63 glycerol objective on a spectral confocal system (TCS SP5; Leica Microsystems). PASMC were identified by positive α-actin fluorescence. Within these cells, percent colocalization of SYTOX (nuclear) and NFATc3 fluorescence was calculated using Leica software and averaged for all pulmonary artery vascular smooth muscle cells within that artery (8–10 arteries per animal).

Generation of PASMC Culture

ASIC1^{+/+} and ASIC1^{−/−} mice were anesthetized with pentobarbital sodium (200 mg/kg ip), and the heart and lungs were removed by midline thoracotomy. Intrapulmonary arteries (approximately 2nd to 5th order) were dissected free, enzymatically digested in reduced-Ca^{2+} HBSS, and diltiazem (Sigma-Aldrich) to prevent Ca^{2+} entry through L-type voltage-gated Ca^{2+} channels, and 10 μM cyclopiazonic acid (CPA) to deplete intracellular Ca^{2+} stores and prevent Ca^{2+} reuptake through the sarcoplasmic reticulum Ca^{2+}-ATPase. The concentrations of ET-1 (10^{-10} to 10^{-8} M; Sigma-Aldrich) in isolated pulmonary arteries from ASIC1^{+/+} and ASIC1^{−/−} mice was assessed as previously described (39). Briefly, fura-2-loaded arteries were superfused with Ca^{2+}-free, HEPES-based PSS (in mM: 130 NaCl, 4 KC1, 1.2 MgSO_{4}, 4 NaHCO_{3}, 10 HEPES, 1.18 KH_{2}PO_{4}, 6 glucose, 3 EGTA; pH adjusted to 7.4 with NaOH) containing 50 μM diltiazem (Sigma-Aldrich) to prevent Ca^{2+} entry through L-type voltage-gated Ca^{2+} channels, and 10 μM cyclopiazonic acid (CPA; Calbiochem) to deplete intracellular Ca^{2+} stores and prevent Ca^{2+} reuptake through the sarcoplasmic reticulum Ca^{2+}-ATPase. The changes in [Ca^{2+}], were determined upon repletion of HEPES-based PSS containing 1.8 mM CaCl_{2} in the continued presence of diltiazem and CPA. Area under the curve (AUC) was calculated as previously described (44).

Determination of NFAT, TRPC1, and Orai1 Expression in PASMC from ASIC1^{+/+} and ASIC1^{−/−} Mice

Messenger RNA expression. Total RNA was isolated from PASMC from ASIC1^{+/+} and ASIC1^{−/−} mice using TRI Reagent (Zymo Research). RNA extracts were treated with DNase I and RNA cleanup was performed using Zymo-Spin IIiGC Columns (Zymo Research) following the manufacturer’s recommendations. Messenger RNA was reverse-transcribed to cDNA using a High Capacity Reverse Transcription kit (Life Technologies). Specific primers (Integrated DNA Technologies) and probes (Roche) were used to detect transcripts for NFATc2, NFATc3, TRPC1, and Orai1 by real-time quantitative PCR. Primer sequences and probe number are listed in Table 1. β-Actin was
Table 1. Primers and probes used for real-time, quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Pair Sequence, Sense/Antisense</th>
<th>Probe Number*</th>
</tr>
</thead>
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<tr>
<td>NFATc2</td>
<td>5'-TGTGAACACCCCAACAGGAG-3'</td>
<td>45</td>
</tr>
<tr>
<td>NFATc3</td>
<td>5'-TGCTGCTGCTGCTGACCTTAAAGGAAC-3'</td>
<td>49</td>
</tr>
<tr>
<td>TRPC1</td>
<td>5'-TGAACCTAAGTGCTGACCTTAAAGGAAC-3'</td>
<td>10</td>
</tr>
<tr>
<td>Orai1</td>
<td>5'-TACCGGTACCGGTCTTCAAATAAAGGAAC-3'</td>
<td>108</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-TCAAGGCCAACCCTGTTGAAAC-3'</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>5'-GACAGGGCGATACGGAAGGGAAC-3'</td>
<td></td>
</tr>
</tbody>
</table>

*Roche.

used as the endogenous control and normalizer of gene expression (dCt). Relative quantification was determined by the Comparative Ct method (2^{ΔΔCt}) in which gene expression was determined relative to a single calibrator from an ASIC1+/− mouse (33).

Protein expression. PASMC from ASIC1+/− and ASIC1−/− mice were homogenized in 10 mM Tris-HCl homogenization buffer (containing 255 mM sucrose, 12 mM EDTA, 12 μM leupeptin, 1 μM pepstatin A, and 0.3 μM aprotinin) and centrifuged at 10,000 g for 10 min at 4°C to remove insoluble debris. Sample protein concentrations were determined by the Bradford method (Bio-Rad). PASMC lysates (30 μg) were separated by SDS-PAGE (7.5% or 12% TGX; BioRad) and transferred to polyvinylidene difluoride membranes. Blots were blocked for 1 h with 5% milk and then incubated overnight at 4°C with rabbit anti-TRPC1 (1:250, Abcam) or rabbit anti-Orai1 (1:250, Alomone Labs) and subsequently for 1 h with rabbit anti-GAPDH (1:5,000, Sigma Aldrich). For immunohistochemical labeling, blots were incubated for 1 h with goat anti-rabbit IgG-horseradish peroxidase (1:3,000, Bio-Rad). Blots were exposed to chemiluminescence-sensitive film (GeneMate) and quantification of TRPC1, Orai1, and GAPDH bands was accomplished by densitometric analysis of scanned images (Sigma Gel).

Determination of ASIC1-PICK1-Calcineurin Colocalization

Protein-protein interactions in PASMC were determined using the Duolink in situ Proximity Ligation Assay (PLA) according to the manufacturer’s instructions (Olink Biosciences, Sigma Aldrich) as previously described (14). Briefly, PASMC were plated on 18-well slides (Ibidi) and grown until 75% confluent. PASMC were fixed with 2% paraformaldehyde and incubated with Duolink blocking slides (Ibidi) and grown until

500 m at 37°C to remove insoluble debris. Sample protein concentrations were determined by the Bradford method (Bio-Rad). PASMC lysates (30 μg) were separated by SDS-PAGE (7.5% or 12% TGX; BioRad) and transferred to polyvinylidene difluoride membranes. Blots were blocked for 1 h with 5% milk and then incubated overnight at 4°C with rabbit anti-TRPC1 (1:250, Abcam) or rabbit anti-Orai1 (1:250, Alomone Labs) and subsequently for 1 h with rabbit anti-GAPDH (1:5,000, Sigma Aldrich). For immunohistochemical labeling, blots were incubated for 1 h with goat anti-rabbit IgG-horseradish peroxidase (1:3,000, Bio-Rad). Blots were exposed to chemiluminescence-sensitive film (GeneMate) and quantification of TRPC1, Orai1, and GAPDH bands was accomplished by densitometric analysis of scanned images (Sigma Gel).

Determination of NFATc3 Nuclear Import

PASMC from intrapulmonary arteries of ASIC1+/− and ASIC1−/− mice were cultured until confluent (approximately 5–8 days) at 37°C with 6% CO2. PASMC were electroporated with NFATc3-enhanced green fluorescent protein (EGFP) expression vector using Nucleofector (Lonza). The vector was created by Dr. F. McKeon (Harvard University, Cambridge, MA) and kindly provided by Dr. L.F. Santana (Washington State University, Seattle, WA). NFATc3-EGFP-expressing PASMC were seeded on microscope coverslips and differentiated by culturing for at least 48 h in Serum-Free Smooth Muscle Cell Medium (Cell Biologics). Cells were incubated for 30 min with a myosin light chain peptide inhibitor (1 μM, Peptide 18; Calbiochem) in HEPES-PSS to reduce cell shrinkage in response to constrictors. Experiments were conducted in the presence or absence of the ASIC1 inhibitor PeTX1 (20 nM) or the PICK1 inhibitor FSC231 (50 μM). Treatment was initiated with the addition of vehicle, ET-1 (10−8 M, Sigma Aldrich), or ionomycin (10−7 M, Calbiochem) and the CRM1-exporting inhibitor leptomycin B (4 × 10−8 M, LC Laboratories), which prevents NFATc3-EGFP nuclear export. After 30 min of treatment, cells were fixed with 4% formaldehyde in PBS, washed with PBS, and imaged using a ×63 objective on a Leica TCS SP5 Spectral Confocal System. Nuclear (Fn) and cytosol EGFP fluorescence (Fc) were measured by placing regions of interest in the nucleus and cytosol with the Leica LAS AF Lite software, and fluorescence values were background-corrected and expressed as Fc/Fn. Similarly, NFATc3-EGFP nuclear translocation was determined in PASMC from ASIC1+/− and ASIC1−/− mice stimulated with ET-1 or ionomycin.

Calculations and Statistical Analyses

Results are expressed as means ± SE. Values of n refer to number of animals in each group unless otherwise stated. Statistical significance was tested at the 95% (P < 0.05) confidence level using an unpaired t-test, one-way ANOVA, or two-way ANOVA followed by a Student-Newman-Keuls posttest. The statistical test performed for each experiment is indicated in the figure legends.

RESULTS

CH-mediated increases in basal [Ca2+]i and SOCE in small pulmonary arteries are dependent on ASIC1. We have previously demonstrated that the only NFAT isoform activated by CH in mouse pulmonary arteries is NFATc3 (9). NFATc3 activation was time-dependent, with 1 wk of CH being the time with the highest percent of PASMC with nuclear NFATc3 (9). Therefore, we chose this time to examine the contribution of ASIC1 Ca2+ influx to NFATc3 nuclear translocation and activation. Consistent with our previous data following 4 wk of CH (39), we demonstrate that baseline fura-2 ratios (Fig. 1A) and SOCE (Fig. 1, B and C) were greater in arteries isolated from ASIC1+/− mice exposed to 1 wk of CH compared with control ASIC1+/+ mice. This CH-mediated increase in basal [Ca2+]i and SOCE was not present in arteries isolated from ASIC1−/− mice (Fig. 1). Furthermore, SOCE responses from control and CH ASIC1−/− mice were diminished compared with respective ASIC1+/+ mice (Fig. 1, B and C). These data,
along with previous observations from our laboratory (21, 39), demonstrate the involvement of ASIC1 in SOCE in control animals as well as the augmentation of this response after exposure to CH.

ASIC1 contributes to CH-induced NFATc3 nuclear accumulation in PASMC. To determine the role of ASIC1 in NFATc3 nuclear translocation in PASMC, we examined the colocalization of anti-NFATc3 and SYTOX. CH exposure increased colocalization of anti-NFATc3 and SYTOX in PASMC from ASIC1+/+ mice but not ASIC1−/− mice (Fig. 2). These data are consistent with our previous report in BALB/c and FVBN mice showing CH-induced increases in NFATc3 nuclear localization (9), and suggest that ASIC1 is important in hypoxia-induced NFATc3 nuclear localization and activation.

ET-1 vasoconstrictor and arterial wall [Ca2+]i responses are impaired in ASIC1−/− mice. Activation of the ET-1 system has been demonstrated in patients with pulmonary hypertension and in animal models of pulmonary hypertension (13). We previously demonstrated that ASIC1 contributes to ET-1-induced pulmonary arterial vasoconstriction (21, 39) and that ET-1 contributes to CH-induced NFATc3 activation in mouse pulmonary arteries (8). ET-1-induced vasoconstrictor responses were largely blunted in arteries from both control (Fig. 3A) and CH (Fig. 3B) ASIC1−/− mice when compared with ASIC1+/+ mice. Changes in arterial wall [Ca2+]i were similarly diminished in isolated arteries from ASIC1−/− mice compared with arteries from ASIC1+/+ mice (Fig. 3, C and D). There was no significant difference in ET-1-induced vasoreactivity following 1 wk of CH compared with controls in either group. Although 1 wk of CH exposure tended to increase ET-1-mediated vasoconstrictor reactivity in arteries from ASIC1+/+ mice (Fig. 3, A and B), 1 wk may not be a sufficient length of CH exposure to observe a significant augmentation of ET-1-mediated vasoconstriction as we have previously shown in arteries from ASIC1+/+ mice following 4 wk of CH (39).

ASIC1 contributes to ET-1-induced increases in PASMC [Ca2+]i and NFATc3 nuclear import. To better understand the mechanism by which ASIC1 contributes to activation of NFATc3, we examined ET-1-induced NFATc3 nuclear localization in cultured PASMC from ASIC1+/+ and ASIC1−/− mice. We first examined the contribution of ASIC1 to ET-1-induced Ca2+ influx in cultured mouse PASMC. ET-1 resulted in a rapid increase in [Ca2+]i that stabilized at lower levels (Fig. 4A). This initial [Ca2+]i peak amplitude was unaffected by inhibition of ASIC1 and presumably mediated by release of Ca2+ from the sarcoplasmic reticulum (28). However, the duration of this peak and the subsequent sustained Ca2+ influx was blunted by inhibition of ASIC1. When assessing AUC, the ASIC1 inhibitor psalmotoxin I (PcTX1) significantly diminished ET-1-induced increases in [Ca2+]i in PASMC from ASIC1+/+ mice (Fig. 4B). ASIC1−/− mice similarly showed reduced ET-1-induced Ca2+ entry (Fig. 4B).

Consistent with previous studies (8), NFATc3-EGFP translocated to the nucleus in response to ET-1 in PASMC from ASIC1+/+ mice (Fig. 5, A and B). PcTX1 prevented this ET-1-induced NFATc3 nuclear import in ASIC1+/+ PASMC. We additionally confirmed the data obtained using a pharmacologic ASIC1 inhibitor by showing that ET-1-induced NFATc3 nuclear import was significantly attenuated in cultured PASMC from ASIC1−/− mice compared with PASMC from ASIC1+/+ mice. To specifically determine whether Ca2+ influx through ASIC1 is important in activating NFATc3, we induced NFATc3 nuclear import by increasing [Ca2+]i, with ionomycin, a calcium ionophore. Under these experimental conditions in which we have bypassed Ca2+ influx through ASIC1, there was no difference in NFATc3 nuclear import between ASIC1+/+ and ASIC1−/− PASMC (Fig. 5C). These experiments demonstrate that downstream of Ca2+ entry, signaling of NFATc3 nuclear import is intact in PASMC from ASIC1+/+ mice and further highlight the essential role for
ASIC1-mediated Ca\(^{2+}\) influx in both CH- and ET-1-induced NFATc3 nuclear translocation.

Knockout of ASIC1 does not alter NFAT, Orai1, or TRPC1 expression in PASMC. We have shown that NFATc3, but not NFATc2, is activated in PASMC by hypobaric CH (8, 9). However, NFATc2 has been shown to be activated in PASMC from patients with primary pulmonary arterial hypertension and in other animal models of pulmonary hypertension in a Ca\(^{2+}\)-dependent and independent manner (35, 41). Although the NFATc3 nuclear import pathway in response to ionomycin is not impaired in PASMC from ASIC1\(^{-/-}\) mice (Fig. 5C), we further sought to determine whether absence of the ASIC1 gene affects NFATc2 or NFATc3 mRNA expression. There was no difference in NFATc2 (Fig. 6A) or NFATc3 (Fig. 6B) mRNA levels between ASIC1\(^{-/-}\) and ASIC1\(^{+/+}\) PASMC. These data further suggest that the underlying mechanism of NFATc3 activation in PASMC is Ca\(^{2+}\) influx, and not a change in NFAT isoform expression.

Several store-operated channels in PASMC contribute to development of pulmonary hypertension (46). To determine whether the knockout of ASIC1 alters the expression level of other store-operated channels, we examined mRNA and protein expression of Orai1 and TRPC1. There was no difference in Orai1 (Fig. 6, C and E) or TRPC1 (Fig. 6, D and F) mRNA or protein expression between ASIC1\(^{-/-}\) and ASIC1\(^{+/+}\) PASMC. Although these data suggest other store-operated

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**Fig. 2.** ASIC1 contributes to CH-induced NFATc3 nuclear accumulation. Representative images (A) and summary data (B) showing NFATc3 nuclear translocation (white) was assessed by % NFATc3 colocalization (red) with SYTOX nuclear stain (green) in smooth muscle α-actin-positive cells (blue) in fixed lung sections from control and CH-exposed (1 wk) ASIC1\(^{+/+}\) and ASIC1\(^{-/-}\) mice. Data are expressed as means ± SE, n = 32 arteries from 4 animals per group. *P < 0.05 vs. control mice, #P < 0.05 vs. ASIC1\(^{+/+}\) mice analyzed by a two-way ANOVA and individual groups compared with the Student-Newman-Keuls test.

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**Fig. 3.** ASIC1 contributes to ET-1-mediated vasoconstriction in isolated, pressurized small pulmonary arteries. Vasoconstriction (percent baseline diameter, A and B) and changes in arterial wall [Ca\(^{2+}\)] (ΔF\(_{340/380}\)/F\(_{380}\), C and D) in response to endothelin-1 (ET-1, 10\(^{-10}\) to 10\(^{-8}\) M) in small pulmonary arteries from control (A and C) and CH-exposed (1 wk) (B and D) ASIC1\(^{+/+}\) and ASIC1\(^{-/-}\) mice. Values are means ± SE, n = 5–7 animals/group. *P < 0.05 vs. ASIC1\(^{+/+}\) mice analyzed by a two-way ANOVA and individual groups compared with the Student-Newman-Keuls test.

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ASIC1 activation of NFATc3

channels are not compromised by the absence of ASIC1, we do not currently know whether ASIC1 interacts with these other molecules to form a store-operated signaling complex.

**Colocalization between ASIC1, PICK1, and calcineurin B.** NFATc3 is regulated by Ca\(^{2+}\) and calcineurin, a Ca\(^{2+}\)/calmodulin-dependent serine phosphatase. In rat PASMC we have recently shown a functional association between ASIC1 and PICK1/calcineurin (14), however, the physical interaction is unknown. Using the Duolink PLA we observe colocalization of ASIC1 with PICK1 and calcineurin B in PASMC from ASIC1\(^{+/+}\) mice, as demonstrated by red puncta (Fig. 7A). This interaction was absent in PASMC from ASIC1\(^{-/-}\) mice (Fig. 7B). PICK1 and calcineurin B colocalize in PASMC from both ASIC1\(^{+/+}\) and ASIC1\(^{-/-}\) mice (Fig. 7A and B). As a negative control, either both primary antibodies were omitted (not shown) or one primary antibody was omitted at a time. We observed very few, if any, puncta under these conditions (Fig. 7C).

**PICK1 is essential for NFATc3 nuclear import.** Considering the possibility that PICK1 provides the scaffold that localizes ASIC1 and calcineurin B, we examined the role of PICK1 in NFATc3 nuclear import in PASMC from ASIC1\(^{+/+}\) mice. ET-1 resulted in increased NFATc3 nuclear translocation in PASMC from ASIC1\(^{+/+}\) mice that was prevented by the PICK1 PDZ-domain inhibitor FSC231 (Fig. 8A). FSC231 also diminished NFATc3 nuclear import in response to ionomycin, a calcium ionophore (Fig. 8B), suggesting that PICK1 may contribute to NFATc3 nuclear import downstream of Ca\(^{2+}\) influx. To specifically determine whether PICK1 influences Ca\(^{2+}\) entry in mouse PASMC from ASIC1\(^{+/+}\) mice, we examined the effect of FSC231 on changes in \([\text{Ca}^{2+}]_i\) in responses to ET-1 and SOCE. FSC231 did not alter Ca\(^{2+}\) influx to either ET-1 (Fig. 9A) or SOCE (Fig. 9B). These data suggest that PICK1 may facilitate NFATc3 nuclear translocation downstream of ASIC1-mediated Ca\(^{2+}\) influx.

**DISCUSSION**

Our research group has previously demonstrated that CH activates both ASIC1 (21, 39) and NFATc3 (8, 9) in PASMC, which contributes to the development of pulmonary hypertension. The goal of this study was to determine whether ASIC1-mediated Ca\(^{2+}\) influx contributes to CH- and ET-1-induced...

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**Fig. 4.** ASIC1 contributes to ET-1-induced Ca\(^{2+}\) influx in mouse PASMC. A: representative traces showing the change in fura-2 ratio. B: summary data of area under the curve (AUC) in response to ET-1 in mouse pulmonary arterial smooth muscle cell (PASMC) from ASIC1\(^{+/+}\) mice in the presence or absence of psalmotoxin 1 (PcTX1, 20 nM) or ASIC1\(^{-/-}\) mice. Values are means ± SE, n = 4–6/group. *P < 0.05 vs. ASIC1\(^{+/+}\) mice analyzed by a one-way ANOVA and individual groups compared with the Student-Newman-Keuls test.

**Fig. 5.** ASIC1 Ca\(^{2+}\) influx contributes to ET-1-induced NFATc3 nuclear import. A: mouse PASMC from ASIC1\(^{+/+}\) or ASIC1\(^{-/-}\) mice were transfected with NFATc3-enhanced green fluorescent protein (EGFP), EGFP nuclear (Fn)/cytosolic fluorescence (Fc) was determined in PASMC treated with vehicle, ET-1 (100 nM, B), or ionomycin (0.1 μM, C). PASMC from ASIC1\(^{+/+}\) mice were additionally treated with PcTX1 (20 nM, A and B). Values are means ± SE, n = 3 replicates/group from average of 20–25 cells/each replicate. *P < 0.05 vs. vehicle, #P < 0.05 vs. ASIC1\(^{-/-}\) mice analyzed by a two-way ANOVA and individual groups compared with the Student-Newman-Keuls test.
ASIC1 activation of NFATc3

NFATc3 activation in mouse PASMC. Consistent with our previous studies, we found that 1-wk CH-mediated increases in basal [Ca\(^{2+}\)] and SOCE in small pulmonary arteries are dependent on ASIC1. The key findings of the present work are that 1) ASIC1-mediates CH- and ET-1-induced NFATc3 nuclear import, and 2) the scaffolding protein PICK1 is essential for NFATc3 nuclear import. Together, these data indicate that ASIC1 and PICK1 are both major contributors to CH- and ET-1-induced NFATc3 nuclear import. Furthermore, these data provide an essential link between CH-induced ASIC1-mediated Ca\(^{2+}\) influx, activation of the NFATc3 transcription factor, and the development of pulmonary hypertension.

Regulation of NFAT by Ca\(^{2+}\) and the Ca\(^{2+}\)/calmodulin-dependent serine phosphatase calcineurin provides a direct link between intracellular Ca\(^{2+}\) signaling and gene expression. Additionally, activation of some NFAT isoforms can occur independent of Ca\(^{2+}\) through regulation of NFAT expression. Both the signal transducers and activators of transcription-3 (STAT3)/Pim-1 and epigenetic reader bromodomain-containing protein-4 (BRD4), which have binding sites on the NFAT promoter region, have been implicated in pulmonary arterial hypertension (35, 41). However, we have previously demonstrated that the only NFAT isoform activated by hypobaric hypoxia or oxidative stress in PASMC is NFATc3, and the mechanism does not involve upregulation of its expression (9, 45). Consistent with our previous findings, NFATc2 and NFATc3 mRNA levels were similar in PASMC from ASIC1\(^{+/+}\) and ASIC1\(^{−/−}\) mice, suggesting that decreased NFATc3 nuclear translocation in PASMC from ASIC1\(^{−/−}\) mice is not due to downregulation of NFATc3 expression. Moreover, we observe similar inhibition of NFATc3 nuclear import when we acutely inhibit ASIC1 with PcTX1. Therefore, it is unlikely that ASIC1 regulates NFATc3 activation at the level of transcription/translation; rather, our data support a role for ASIC1-dependent Ca\(^{2+}\) influx in activation of NFATc3 in PASMC.

It was recently shown that signaling in specific microdomains is more important for nuclear translocation of NFAT than global increases in [Ca\(^{2+}\)]. (26). It is well established that NFAT is activated by cell-surface receptors coupled to SOCE and/or Ca\(^{2+}\)-release-activated Ca\(^{2+}\) (CRAC) in a variety of cell types, including vascular smooth muscle (3, 18, 42, 52). In patients with a rare form of hereditary severe combined immunodeficiency, T cells display a pronounced reduction in SOCE, which leads to a selective inability to activate all NFATs (12). SOCE is a dynamic and highly regulated process that is known to be mediated by two distinct types of ion currents. The CRAC current displays high selectivity for Ca\(^{2+}\) and is mediated by the Orai1 channel (29). Store depletion also activates nonselective cation channel currents (1, 55), believed to be mediated by various transient receptor potential (TRP) channels. The depletion of Ca\(^{2+}\) is sensed by the Ca\(^{2+}\)-binding protein stromal interaction molecule-1 (STIM1), which then binds to and activates store-operated channels (32, 48). In PASMC, STIM1 interacts with both Orai1 and the classical/canonical TRP-1 (TRPC1) channel (36, 37). In HEK293 and human submandibular gland cells, Orai1-mediated Ca\(^{2+}\) influx induces nuclear translocation of NFAT and NFAT-dependent gene expression; however, global increases in [Ca\(^{2+}\)] or Ca\(^{2+}\) influx through TRPC1 or TRPC3 was not sufficient to activate NFAT (25, 40). One possible reason for this contrasting response is the association of these ion channels with necessary scaffolding proteins. Following store depletion, Orai1 assembles in a membrane-delimited signaling complex with calcineurin and calmodulin, via the scaffolding protein A kinase-anchoring protein 79/150 (AKAP79/150 (27)). By contrast, the related Orai3 interacts poorly with AKAP79/150 and fails to activate NFAT (27). Together, these studies demonstrate that discrete Ca\(^{2+}\) events can differentially activate NFAT.

On the basis of current data and previous data from our laboratory (14, 21, 22, 39, 44), ASIC1 appears to be a major component, but unlikely the only component of SOCE in PASMC. How ASIC1 contributes to the SOCE response and its interactions with other known mediators of SOCE such as STIM1, TRPC1, or Orai1 is of interest and an area we are currently investigating. ASIC1 does, however, interact with
several scaffolding proteins such as AKAP/150 and PICK1 and with the phosphatase calcineurin (4, 11, 14, 17). Therefore, the goal of this study was to determine whether ASIC1-mediated SOCE is involved in activation of NFATc3 in PASMC.

In our previous studies, administration of the L-type Ca\(^{2+}\)/H11001 channel inhibitor diltiazem attenuated but did not abolish CH-induced NFATc3 activation, suggesting that other Ca\(^{2+}\)-influx pathways contribute to increased NFATc3 nuclear translocation (8). Our first key finding in the present work is that ASIC1-mediated Ca\(^{2+}\) influx is essential for both hypoxia- and ET-1-induced NFATc3 nuclear translocation. The findings supporting Ca\(^{2+}\) influx over other means of NFAT activation are as follows: 1) NFATc3 expression is not altered in PASMC from ASIC1\(^{-/-}\) mice compared with ASIC1\(^{+/+}\) mice, 2) CH-mediated increases in basal [Ca\(^{2+}\)], and SOCE in small pulmonary arteries are dependent on ASIC1, 3) inhibition of ASIC1 largely attenuated ET-1-induced Ca\(^{2+}\) responses, and 4) bypassing ASIC1-mediated Ca\(^{2+}\) influx by using ionomycin resulted in similar NFAT nuclear import between ASIC1\(^{+/+}\) and ASIC1\(^{-/-}\) PASMC. Previous studies showing that ASIC1 interacts with calcineurin (4), and our current finding of colocalization of ASIC1 and calcineurin B provide additional

Fig. 7. Colocalization of ASIC1, PICK1, and calcineurin B in mouse PASMC. Representative confocal images of the Duolink PLA interaction (red puncta) between ASIC1-PICK1 (left), ASIC1-calcineurin B (middle), and PICK1-calcineurin B (right) in PASMC from ASIC1\(^{+/+}\) mice (A) and ASIC1\(^{-/-}\) mice (B). Proximity Ligation Assay (PLA) interactions are not detected for ASIC1-PICK1 or ASIC1-calcineurin B in PASMC from ASIC1\(^{-/-}\) mice. For negative controls (C), PASMC were incubated with each primary alone and both PLA probes. Actin is labeled with Alexa Fluor 647 phalloidin (blue) and the nuclei are labeled with SYTOX (green).
vehicle

Ca2+ influx in mouse PASMC, as we previously had in rat PASMC. Other than species differences, the reason for the disparities is support for this pathway. Furthermore, ASIC1 is involved in acid-stimulated NFATc1 signaling in osteoclastogenesis. Acid-induced increases in [Ca2+]i to activate calcineurin/NFATc1 signaling is the major stimulus of osteoclast differentiation, and ASIC1a activation in rat osteoclasts induces NFATc1 nuclear translocation and transcriptional activity (31). We anticipate ASIC1 is activated by agonist-induced store-depletion in PASMC, although we cannot currently discount that CH causes localized changes in H+ resulting in activation of ASIC1.

The laboratory headed by R. Simon (4) reported that PKA, in association with AKAP150, is required for full activation of ASICs in neurons and CHO cells. Our recent study using rat PASMC showing that PKA phosphorylates ASIC1 and stimulates channel activity is consistent with this previous study (14). Although the interaction between PICK1 and ASIC1 is well characterized, the functional significance of this interaction is not well understood. PICK1 has been shown to increase ASIC1 surface expression and clustering (11, 17, 24); however, our recent findings suggest that PICK1 interaction with ASIC1 is associated with changes in the phosphorylation state of the channel rather than plasma membrane localization. More specifically, ASIC1 activity was inhibited by a PICK1-dependent and calcineurin-mediated dephosphorylation of ASIC1 (14). Simon’s laboratory (4) also reported association with calcineurin in a dephosphorylation and inactivation of ASIC1. Although both AKAP150 and PICK1 also associate with calcineurin (5, 10, 19) and have been shown to promote NFAT activity in various cell types (19, 30, 38), these previous findings regarding reciprocal modulation of ASIC1 activity by PKA/AKAP150 and PICK1/calcineurin directed our focus on PICK1. Interestingly, in the current study we did not observe the same inhibitory effect of PICK1 on ASIC1-mediated Ca2+ influx in mouse PASMC, as we previously had in rat PASMC. Other than species differences, the reason for the disparities is...
currently unknown. However, the lack of PICK1-mediated inhibition on ASIC1-mediated Ca\(^{2+}\) influx in mouse PASMC allowed us to more accurately study the role of ASIC1 and PICK1 in downstream activation of NFATc3.

The second key finding of the present study is that PICK1 is necessary for ET-1-stimulated NFATc3 nuclear import. The scaffolding protein PICK1 mediates the direct interaction of proteins containing PDZ binding motifs. In addition to the PDZ domain, PICK1 contains a larger Bin/amphiphysin/Rvs (BAR) domain, which binds to lipids to facilitate membrane localization (23). Both the PDZ and BAR domains are essential for vesicular formation and protein trafficking (23, 24, 43). The scaffolding properties of PICK1 enable macromolecular complexes to form, assembling molecules into signaling domains. PICK1 associates with ASIC1 (11, 17) and with calcineurin, through which PICK1 promotes activation of NFAT in PC12 cells (19). Our data suggest that PICK1 has several functions. First, PICK1 provides the necessary scaffolding for ASIC1 and calcineurin, bringing them in close proximity to promote activation of calcineurin by ASIC1-mediated Ca\(^{2+}\) influx, potentially in conjunction with AKAP79/150. Second, PICK1 appears to participate in the Ca\(^{2+}\)-independent activation and/or trafficking of calcineurin-NFATc3 to the nucleus. This is supported by the data demonstrating that PICK1 inhibition diminished NFATc3 nuclear accumulation in response to both ET-1 and ionomycin, but did not alter ET-1-induced Ca\(^{2+}\) influx or SOCE in PASMC. Following Ca\(^{2+}\) influx, PICK1 may facilitate the interaction of ASIC1 and calcineurin and downstream activation and nuclear translocation of NFATc3.

In addition to Ca\(^{2+}\)-dependent activation of calcineurin, ET-1-induced NFATc3 nuclear translocation requires RhoA/Rho kinase (ROCK) and an intact dynamic actin cytoskeleton (8). PICK1 can interact with cytoskeletal proteins but with some controversial outcomes (34, 47). For example, PICK1 was shown to inhibit actin filament (F-actin) nucleation by the Arp2/3 complex through interactions of the PICK1 BAR domain with F-actin (47). More recently, researchers in the laboratory of R. Dominguez (34) confirmed that PICK1 binds F-actin in vitro; however, in cells, PICK1 did not bind directly to F-actin-rich structures, but they used F-actin indirectly for the fast and nondirectional movement of PICK1-associated vesicles. In these studies, the acidic C-terminal tail (ACT) of PICK1 linked PICK1-associated vesicles to a motility factor such as actin-based myosin motor (34). Together, these data argue that NFATc3 nuclear translocation requires PICK1 association and a properly assembled actin cytoskeleton.

In summary, we observed a complex mechanism of NFATc3 activation in mouse PASMC that involves Ca\(^{2+}\) influx through ASIC1 and downstream regulation by the scaffolding protein PICK1 (Fig. 10). It draws together our previous findings that CH activates both ASIC1 and NFATc3. The contribution of ASIC1 to CH-induced pulmonary vascular remodeling further supports an important role for ASIC1-mediated Ca\(^{2+}\) influx in NFATc3 activation of proliferation and hypertrophy in PASMC and the overall development of hypoxic pulmonary hypertension (2, 7, 9, 39). Our findings suggest that ASIC1 acts as a direct signal transducer and may be a potential therapeutic target for modulation of hypoxia-dependent transcription pathways associated with the pathophysiology of hypoxic pulmonary hypertension.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


