Constrictor-induced translocation of NFAT3 in human and rat pulmonary artery smooth muscle

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Yaghi, Asma, and Stephen M. Sims. Constrictor-induced translocation of NFAT3 in human and rat pulmonary artery smooth muscle. Am J Physiol Lung Cell Mol Physiol 289: L1061–L1074, 2005. —The transcription factor nuclear factor of activated T cells (NFAT) resides in the cytoplasm in resting cells and upon stimulation is dephosphorylated, translocates to the nucleus, and becomes transcriptionally active. NFAT is commonly activated by stimulation of receptors coupled to Ca^{2+} mobilization; however, little is known about the regulation of NFAT in pulmonary vascular smooth muscle. The aim of this study was to investigate regulation of NFAT in human and rat intralobar pulmonary artery by two constrictors: phenylephrine (PE) and 20-hydroxyeicosatetraenoic acid (20-HETE), a cytochrome P-450 metabolite formed endogenously in lungs. Immunostaining of smooth muscle cells revealed cytoplasmic localization of NFAT in untreated cells, and PE or 20-HETE induced translocation to the nucleus, with maximal effect at 30 min. Cyclosporin A and FK-506 (both 1 μM) inhibited NFAT translocation, indicating involvement of calcineurin. Moreover, the Rho-kinase blocker Y-27632 prevented translocation. Translocation of NFAT was confirmed by Western blots, with NFAT3 the prominent isoform in pulmonary artery. Constrictors caused calcineurin-sensitive translocation of NFAT to nuclei in intact arteries, demonstrating regulation in native tissue. To investigate a role for Ca^{2+}, cells were loaded with fura-2. Whereas PE caused an acute transient rise of [Ca^{2+}], 20-HETE caused a prolonged low amplitude rise of [Ca^{2+}]. The involvement of Rho-kinase in PE- and 20-HETE-induced NFAT translocation in pulmonary artery suggests a level of control not previously recognized in smooth muscle. Constrictors of the pulmonary vasculature not only cause acute responses but also activate NFAT, which may alter gene expression in pulmonary health and disease.

transcription factor; Western blot; Rho kinase; phenylephrine; 20-HETE; nuclear factor of activated T cells

THE TRANSCRIPTION FACTOR NFAT (nuclear factor of activated T cells), first discovered in T lymphocytes (32, 36), activates gene transcription in nonimmune cell types as well, including smooth, skeletal, and cardiac muscles (19, 20, 34). Five isoforms of NFAT have been identified, with these four being regulated by Ca^{2+} and the Ca^{2+}/calmodulin-dependent phosphatase calcineurin: NFAT1 (NFAT c2/p), NFAT2 (NFAT c1/c), NFAT3 (NFAT c4), and NFAT4 (NFATc3/x) (5, 22).

NFAT is maintained in an inactive state in the cytoplasm in a phosphorylated form. Elevation of cytoplasmic free Ca^{2+} concentration ([Ca^{2+}]) stimulates calcineurin, which dephosphorylates multiple serine residues, exposing a nuclear localization site that permits translocation of NFATc proteins to the nucleus. NFAT together with other transcription factors binds to DNA and alters gene transcription (5, 32). A number of serine-threonine kinases, including casein kinase I and glyco- gen synthase kinase 3, rephosphorylate NFAT, promoting its return to the cytosol and inactivation of its transcriptional activity (5, 32).

Activation of NFAT occurs in response to stimulation of smooth muscle by diverse agonists, including those acting on G protein-coupled receptors (GPCR) and growth factor receptors (13, 37). Interestingly, the first report of NFAT expression and translocation in vascular smooth muscle described the synergistic activation of NFAT by agonists at GPCR and by platelet-derived growth factor (PDGF) (4). NFAT proteins have since been shown to play critical roles in the vasculature, both at early developing and mature stages (15, 19). Disruption of NFATc3 and NFATc4 genes produces lethal defects in vascular patterning in the embryo, reflecting deficiencies in vessel assembly (15). The canonical pathway for activation of NFAT involves elevation of [Ca^{2+}], and stimulation of calcineurin, but compelling evidence also points to control of export from the nucleus as an important contributor to NFAT residence in the nucleus (12).

Smooth muscle-specific expression of NFAT isoforms has been described. NFAT1 and NFAT2 were the first reported in thoracic aortic smooth muscle cells from rat, based on immunocytochemistry and Western blotting of whole cell lysates (4). More recently human aortic smooth muscle was shown to express NFAT2 and 4, but no immunostaining was evident for NFAT1 or 3 (39). Extensive studies of the cerebral vasculature reveal NFAT3 and 4, but not NFAT1 or 2 (13, 14). Not only is there diversity in expression of NFAT proteins, but different stimuli can exert selective actions on NFAT isoforms. For example, elevation of [Ca^{2+}] with ionophore causes translocation of NFAT2 but not NFAT1 in aortic muscle (4). UTP, acting on P2 nucleotide receptors, causes greater NFAT4 activation than does PDGF (13) even though both agonists mobilize Ca^{2+} from the sarcoplasmic reticulum. In addition, intraluminal pressure is a stimulus for NFAT4 activation in pressurized cerebral arteries (14), implicating NFAT as an important point of convergence for modulating vascular responsiveness.

The pulmonary circulation plays a critical role in gas exchange in the lung, with constriction of the small intralobar arteries vital for balancing flow with demand (29, 41). Pulmonary arteries constrict in response to a number of mediators, including α-adrenergic agonists and 20-hydroxyeicosatetraenoic acid (20-HETE), a cytochrome P-450 metabolite of arachidonic acid formed endogenously in lungs (25, 44). Many
vasoconstrictors activate phospholipases and increase the synthesis and release of 20-HETE in vascular smooth muscle cells (6, 24, 30). In acute lung injury (e.g., pneumonia) pulmonary vascular contractility in response to constrictors is attenuated within 48 h, suggesting ongoing modulation of muscle function (42, 44). Thus studying the effects of constrictors on pulmonary artery smooth muscle could lead to novel ways of modulating the pulmonary circulation in diseases. The aim of this study was to investigate the regulation of NFAT in rat and human pulmonary artery smooth muscle in response to two constrictor agonists, phenylephrine (PE) and 20-HETE.

MATERIALS AND METHODS

Rat pulmonary artery smooth muscle cell isolation. All procedures were approved by the Council on Animal Care of the University of Western Ontario. Male Sprague-Dawley rats (300–350 g) were killed with pentobarbital (400 mg/kg ip), and heart and lungs removed and perfused through the pulmonary artery with cold Krebs’ bicarbonate solution (mM: 122 NaCl, 4.7 KCl, 2.5 CaCl2, 0.8 MgSO4·7H2O, 1.2 NaH2PO4, 10 glucose, 20 NaHCO3, 0.25 EDTA, 5 HEPES, pH 7.4) gassed with 95% O2/5% CO2. Intraoral arteries (100–400 μm diameter) were dissected, cleaned of adventitia, and cut open, and the endothelium was removed with a cotton-tipped applicator dipped in cold Krebs’ solution. Arteries were cut into small rings and dissociated at 31°C in a gently shaking water bath in dissociation solution with enzyme mix 1 [0.5 mg/ml papain, 0.18 mg/ml 1,4-dithio-L-threitol, 0.003 mg/ml sodium nitroprusside (23), 1 mg/ml BSA] for 20 min, then dissociated with enzyme mix 2 (1.2 mg/ml Sigma blend collagenase type F, 1 mg/ml trypsin inhibitor, 0.003 mg/ml sodium nitroprusside, 1 mg/ml BSA) for 15 min and dispersed by trituration with fire-polished Pasteur pipettes. Cells were maintained in primary culture. The dissociation solution used for cell dispersal contained (in mM) 140 NaCl, 4 KCl, 1 MgCl2, 0.05 CaCl2, 10 glucose, and 10 HEPES (pH 7.4 with NaOH).

Human pulmonary arteries. Pulmonary arteries were collected following lung surgery for cancer or lung reduction, in accordance with guidelines of the University of Western Ontario Review Board for Research Involving Human Subjects. Segments of intralobar arteries (1–2 cm diameter) were dissected from sites remote to disease, then placed in ice-cold Krebs’ solution, and transported to the laboratory. Arteries were cleaned of adventitia and endothelium, minced, and then dissociated as described above, but with 1.2 mg/ml Sigma blend collagenase type II.

Smooth muscle cultures. Rat smooth muscle cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Burlington, ON, Canada) supplemented with 10% heat-inactivated FBS, 1% antibiotic (penicillin G sodium, Gibco), and 2 mg/ml sodium bicarbonate (Sigma-Aldrich, Oakville, ON, Canada). Freshly prepared human cells were cultured in DMEM supplemented with 15% heat-inactivated FBS, 1% NEMEM (MEM nonessential amino acids, Gibco), 2% antibiotic solution, and 2 mg/ml sodium bicarbonate. All cells used in this study were maintained in primary cultures at 37°C in 95% air-5% CO2 and were studied between days 8 and 12 for rat cells and days 18 and 21 for human cells. The identity of smooth muscle cells was confirmed by immunostaining for smooth muscle α-actin (1A4; Dako, Mississauga, ON, Canada).

Treatment protocol. Experiments were carried out at 37°C in 95% air-5% CO2. Smooth muscle cells were treated for 30 min with constrictor agonists or their vehicle controls. Plates were treated with inhibitor or its vehicle for 30 min before treatment with agonist for 30 min. Cyclosporin A (CsA) and FK-506 were dissolved in DMSO and added at 1 μM concentration.

NFAT staining protocol. After treatments, coverslips of smooth muscle cells were fixed immediately with 4% formaldehyde in warmed (37°C) phosphate-buffered saline (PBS, pH 7.4) for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked for 2 h with 1% normal goat serum in PBS. Primary antibodies, rabbit anti-NFATc1 (K-18, which recognizes all four cytoplasmic NFAT proteins) and NFATc4 (H-74, which recognizes NFATc3) (Santa Cruz Biotechnology, Santa Cruz, CA) (1:100 dilution) in 1% normal goat serum in PBS, were applied overnight at 4°C. Secondary antibody, Alexa Fluor 546 goat anti-rabbit IgG (Molecular Probes) (1:200 dilution), was applied for 2 h at room temperature. After washing, the coverslips were mounted (Vecta-shield mounting medium, Vector Laboratories) and examined under a Carl Zeiss LSM 510 laser scanning confocal microscope. In intact pulmonary arteries, the same protocol was followed with the addition of nuclear staining with SYTO-13 (10 μM) (Molecular Probes). Red fluorescence of NFAT was measured at an excitation wavelength of 553 nm and emission of 569 nm. Green fluorescence of nuclei stained with SYTO-13 was measured at an excitation wavelength of 488 nm and emission of 509 nm. Specificity of immune staining was confirmed by the absence of fluorescence in coverslips/arteries incubated with primary or secondary antibodies alone.

Immunofluorescence. Muscle cells were plated on 12-mm coverslips in 35-mm dishes and randomized to different treatments, with three or four coverslips studied for each treatment. For each coverslip, the number of cells and the number of cells with nuclei positive for NFAT staining were counted in three or four randomly chosen, ×40 objective fields, with a minimum of 200 cells counted per treatment (cell number was confirmed by superimposing transmitted light images). We quantified NFAT localization by comparing fluorescence intensity in the cytosol and nuclear compartments. Cells were scored as positive if fluorescence intensity was greater in the nucleus compared with the cytoplasm and expressed as a percentage of the cells. This method would tend to underestimate the number of cells with nuclear NFAT localization but provided an objective measure of translocation. When staining for α-actin, we used the nuclear stain TOTO-3 (2.5 μM) (Molecular Probes, Eugene, OR) to identify all the cells in a field (not shown).

Measurement of intracellular [Ca2+]i. Free [Ca2+]i, was determined by cells with 1.5 μM fura-2-acetoxyethyl ester (fura-2 AM, Molecular Probes) for 30 min at room temperature. Cells were placed in a chamber mounted on a Nikon inverted microscope and perfused at 1–3 ml/min at room temperature. Cells were illuminated with alternating 345- and 380-nm light using a Deltascan system (Photon Technology International, London, ON, Canada), with 510-nm emission detected with a photometer. [Ca2+]i was calibrated according to published methods (16). Agonists were applied by pressure ejection from glass micropipettes (Picospritzer II; General Valve, Fairfield, NJ) positioned 50–100 μm from cells.

Nuclear extracts. Vascular muscle was grown to confluence in 100-mm plates, randomized for treatments, and then placed on ice, and media were aspirated. Cells were washed twice with cold TBS (10 mM Tris·HCl pH 8.0, 150 mM NaCl), then scraped into 800 μl of cold TBS, and centrifuged for 15 s at 14000 g at 4°C. Pellets were used for preparing cytoplasmic and nuclear extracts as described previously (3, 35) and frozen at –80°C. The protein content of cell extracts was quantified by the Pierce BCA protein assay (Pierce, Rockford, IL).

Western blotting for NFAT proteins. Equivalent amounts of nuclear proteins were electrophoresed on 10% SDS polyacrylamide gels, and the resolved proteins were transferred onto polyvinylidene difluoride membranes (Amersham Canada, Oakville, ON, Canada). Equal loading was confirmed by staining the gels with Coomassie blue stain. In addition, some membranes were reprobed for β-catenin to evaluate loading of protein and confirmed comparable loading between lanes of nuclear or cytosolic extracts. Antibodies used were as follows: NFATc1 (K-18, sc-1149) rabbit polyclonal antibody (dilution, 1:1,000) which identifies all NFAT proteins; NFATc2 (4G6-G5, sc-7296) mouse monoclonal antibody (dilution, 1:1,000); NFATc1 (7A6, sc-7294) mouse monoclonal...
antibody (dilution, 1:1,000); NFATc4 (H-74, sc-13036) rabbit polyclonal antibody (dilution, 1:2,000; Promega, Madison, WI) and enhanced chemiluminescence (ECL Plus, Amersham Canada) with a prestained protein marker (Promega) on all gels. Jurkat and Ramos whole cell lysates (Santa Cruz Biotechnology) were used as positive controls. For repeat probing, membranes were washed with Restore Western Stripping Buffer (Pierce). Optimal densities of protein bands are expressed in arbitrary units.

Reagents. Antibodies for NFAT were from Santa Cruz Biotechnology; 20-HETE was from Cayman Chemical (Ann Arbor, MI). CsA and FK-506 were from Biomol Research (Plymouth Meeting, PA). Other reagents needed for SDS-PAGE electrophoresis, transfer, and Western blotting of proteins were from Amersham Canada. Blotto in TBS (Pierce) was used for blocking the membranes. All other chemicals were from.

Fig. 1. Phenylephrine (PE) induces translocation of nuclear factor of activated T cells (NFAT) from cytosol to the nuclei of rat pulmonary artery smooth muscle. A: representative images showing NFAT staining with or without PE (10 μM). After treatment, cells were fixed and incubated with rabbit polyclonal antibody NFATc1 (K-18)-R that identifies all NFAT isoforms, and localization was detected by goat anti-rabbit IgG. The absence of fluorescence staining in cells incubated without primary antibody indicates specificity of staining. Control pulmonary artery smooth muscle cells showed little or no staining for NFAT in their nuclei, with staining localized in the cytoplasm. Intensity profiles were determined for regions indicated by the doubleheaded arrows, with intensity plots below demonstrating cytoplasmic localization of NFAT in control cells; 3 nuclei are noted. PE treatment (30 min, plus PE) induced NFAT translocation to the nuclei, as evident in the micrographs and the corresponding intensity plot below. The scale bars represent 50 μm. B: time course of PE-induced nuclear translocation of NFAT (n = 4 rats). Pulmonary artery muscle cells were incubated for the indicated time points with vehicle (control, 0.1% distilled water) or with PE. Maximal NFAT nuclear localization (% of total) occurred after 30-min treatment with PE. ANOVA followed by Bonferroni, *P < 0.05 compared with corresponding control.
Sigma, Calbiochem (San Diego, CA), or BDH (Toronto, ON, Canada). The Rho-kinase inhibitor Y-27632 was from Calbiochem.

Statistics. Values are provided as means ± SE, with sample sizes as stated in the text. Results were compared by ANOVA followed by Bonferroni’s posttest or by paired t-test as indicated in figure legends. A value of P < 0.05 was considered significant.

RESULTS

We studied pulmonary arteries from 23 rats, with preparations on any given day paired for control and treatment groups. Intact whole artery studies were carried out on samples from an additional seven rats, again with controls always paired with...

Fig. 2. PE induced nuclear translocation of NFAT in human pulmonary artery smooth muscle cells. A: representative images showing NFAT staining (red) in pulmonary artery muscle cells at time 0 and after 30 min with or without PE (10 μM). Specificity of immune staining was confirmed by the absence of fluorescence in smooth muscle cells incubated without primary antibody. PE treatment (30 min, plus PE) resulted in NFAT nuclear localization compared with controls (0 min and 30 min). The scale bars represent 50 μm. B: time course is shown below for PE- or vehicle-treated cells, with data presented from 3 replicates each from 2 human arteries. Maximal NFAT nuclear localization occurred after 30-min treatment with PE. ANOVA followed by Bonferroni, *P < 0.05 compared with corresponding control.
treatment groups. Pulmonary arteries were obtained from 12 human samples. We first confirmed the identity of the cultured smooth muscle cells by staining for α-smooth muscle actin, together with nuclear staining with TOTO-3. In smooth muscle cultures from three rat and three human preparations, >96% of nuclei were associated with positive staining for α-smooth muscle actin, confirming the identity of cells and the purity of the preparation.

Immunocytochemical staining for NFAT in control cells showed largely cytoplasmic localization, with little or no NFAT in the nuclei. Treatment with PE (10 μM) caused reproducible translocation of NFAT to nuclei (Fig. 1A) as

![Image](AJP-Lung Cell Mol Physiol • VOL 289 • DECEMBER 2005 • www.ajplung.org)

**Fig. 3.** 20-Hydroxyeicosatetraenoic acid (20-HETE) induces nuclear translocation of NFAT in pulmonary artery smooth muscle cells. **A:** representative images showing NFAT staining in cultured human pulmonary artery smooth muscle cells. Treatment with 20-HETE (30 min, plus 20-HETE, 156 nM) caused nuclear localization of NFAT. The scale bars represent 50 μm. **B:** time course of 20-HETE-induced nuclear translocation of NFAT, with data presented from 3 replicates each from 3 human arteries. Maximal NFAT nuclear localization occurred after 30-min treatment with 20-HETE compared with control (0.1% ethanol). Similar results were obtained in pulmonary artery smooth muscle cells from rats (n = 3–4 rats; shown in graph at right). ANOVA followed by Bonferroni, *P < 0.05 compared with corresponding control.
demonstrated by the arrows superimposed on the images and the corresponding intensity profiles (Fig. 1A, bottom images). Cells were treated with PE or vehicle and fixed at various time points, revealing that the percentage of cells with predominantly nuclear NFAT localization peaked at 30 min, with recovery by 60 min (Fig. 1B). This time course was validated in cultures from four separate rats, and the PE-induced translocation was further confirmed in 19 additional rat cell preparations.

To examine the relevance of this finding we extended the studies to human pulmonary artery smooth muscle cells. Samples of muscle were obtained from biopsy specimens and healthy muscle cells maintained in primary culture. Under control conditions NFAT was largely localized in the cytoplasm (Fig. 2A). Stimulation with PE caused translocation of NFAT to the nuclei, and when quantified as described above, there was significant translocation within 10 min, peaking at 30 min (Fig. 2B). These results are comparable to that observed in rat smooth muscle cells although the onset of the translocation was somewhat faster in human cells. To our knowledge, this is the first demonstration of agonist-activated NFAT translocation in pulmonary artery smooth muscle cells, with the demonstration in both rat and human.

Previous studies have reported NFAT activation in smooth muscle in response to constrictors including UTP, endothelin, angiotensin II, and PDGF. 20-HETE is a P-450 metabolite of arachidonic acid that causes constriction of arteries from a number of important vascular beds, such as pulmonary, cerebral, and renal arteries (1, 10, 44). Changes in 20-HETE levels occur in lung disease and are accompanied by altered vascular reactivity (44). Accordingly, there is benefit in understanding both acute and longer-term effects of 20-HETE on smooth muscles. When human pulmonary artery smooth muscle cells were treated with 20-HETE (156 nM), a concentration established to cause constriction (44), significant translocation of NFAT from the cytosol to the nucleus was observed (Fig. 3A). The time course of translocation was similar to that seen for PE, with peak nuclear localization occurring at 30 min and recovery by 60 min, with similar responses seen in rat cells (Fig. 3B).

To investigate the mechanism of NFAT translocation induced by PE and 20-HETE, we pretreated both human and rat smooth muscle cells with the calcineurin inhibitors CsA and FK-506 (26, 33) for 30 min. We then applied PE or 20-HETE and examined cells after 30 min, the time of the peak NFAT translocation in these cells. Nuclear localization was quantified as described in previous figures, and CsA and FK-506 both significantly inhibited PE- and 20-HETE-induced NFAT translocation (Fig. 4). These findings were replicated in preparations from three rats and two human artery samples. The results support the involvement of calcineurin in pulmonary artery smooth muscle cell responses to constrictors.

Multiple isoforms of NFAT are reported in smooth muscle, with agonists causing isoform-selective activation (4, 19). Thus we next carried out Western blot analysis to validate the immunocytochemistry and explore NFAT isoform(s) in pulmonary artery smooth muscle. We initially used the broadly reactive (pan) antibody employed for the immunocytochemistry, which identifies all NFAT proteins (NFAT1, NFAT2, NFAT3, and NFAT4). Western blotting revealed a prominent 140-kDa band in control cytoplasmic extracts from rat cells, with bands corresponding to those in Jurkat and Ramos controls. Notably, pretreatment of the smooth muscle cells with PE reduced the NFAT levels in the cytoplasmic extracts (Fig. 5A), suggesting NFAT translocation to the nuclei. We next examined nuclear extracts and found a corresponding increase in nuclear NFAT levels after treatment with PE or 20-HETE (Fig. 5B). In addition, pretreatment of cells with CsA (1 μM) reduced nuclear translocation in response to PE and 20-HETE (Fig. 5B), confirming a role for calcineurin in NFAT activation. Thus Western blot analysis, which was replicated in three independent experiments, confirmed the immunostaining described above. When blots were reprobed for specific NFAT isoforms, we found no indication of NFAT1, 2, or 4 in any of the treatment groups or in the cytosol of pulmonary artery smooth muscle. However, probing for NFAT3 yielded clear bands at ~140 kDa, with significant CsA-sensitive translocation of NFAT in response to both constrictors. Control lanes of Jurkat and Ramos cells confirmed specific isoforms. We note that multiple bands were present in Ramos and Jurkat whole cell extracts, which may represent phosphorylated and unphosphorylated forms of NFAT. However, such multiple bands were not evident in the smooth muscle extracts. These data were verified in three cultures from three rats. When analyzed by densitom-

Fig. 4. Cyclosporin A (CsA) and FK-506 inhibit PE- and 20-HETE-induced NFAT nuclear translocation. In rat (n = 3) and human pulmonary artery (n = 2) smooth muscle cell cultures, pretreatment for 30 min with CsA or FK-506 (both 1 μM) inhibited PE- and 20-HETE-induced NFAT nuclear translocation, indicating the involvement of calcineurin. ANOVA followed by Bonferroni, *P < 0.01 compared with corresponding control, #P < 0.01 compared with corresponding constrictor.
translocation of NFAT3 to the nuclei in rat and human cells (Fig. 6). This effect was blocked by pretreatment with CsA (see bottom panels in Fig. 6, summarization at right).

To investigate the physiological relevance of these findings, we examined constrictor effects on NFAT localization in intact rat pulmonary arteries. Arterial segments were isolated, treated with PE or 20-HETE for 30 min, fixed, stained for NFAT and nuclei, and mounted for confocal microscopy. The schematic representative of an artery segment is shown in Fig. 7A, with smooth muscle and endothelial layers distinguished by their characteristic morphology when imaged at different layers. In this example the artery was treated for 30 min with 20-HETE (156 nM) and stained for NFAT (red) and SYTO-13 (green). A merged image of two slices illustrates the circular orientation of the smooth muscle and the axial orientation of the endothelium. Colocalization of the staining (yellow) indicates that NFAT had translocated to the smooth muscle cell nuclei in response to the constrictor. In contrast, a control vehicle-treated artery showed only diffuse cytoplasmic NFAT staining, with little colocalization evident in the nuclei (Fig. 7B). Higher magnification confocal images taken specifically in the smooth muscle layers reveal the localization of NFAT in the nucleus only after treatment with a constrictor and are shown in Fig. 8 for a PE-treated artery. These results are representative of four independent experiments of three to five arteries each from four rats. In addition, constrictor-induced nuclear translocation of NFAT3 was verified in three independent experiments of three to five arteries each from three rats using the selective NFAT3 antibody. Pretreatment of whole arteries with CsA for 30 min before PE or 20-HETE, resulted in inhibition of the nuclear translocation of NFAT3 (Fig. 9). The inhibitory effect of CsA on constrictor-induced translocation was observed in intact arteries by use of the selective NFAT3 antibody and the pan antibody, as illustrated in representative images for PE (Fig. 9, top) and 20-HETE (Fig. 9, bottom) respectively. Therefore, the results obtained in intact pulmonary arteries reinforce the NFAT localization studies performed on cultured smooth muscle cells and the Western blotting data on cell extracts.

Because NFAT is typically activated by stimulation of receptors coupled to Ca\(^{2+}\) mobilization (32, 40), we investigated changes of [Ca\(^{2+}\)]\(_i\) using the calcium-sensitive dye fura-2 and microspectrophotometry. Whereas acute treatment with 20-HETE caused little change in [Ca\(^{2+}\)]\(_i\) in rat pulmonary artery smooth muscle cells (Fig. 10A), however, functional studies of muscle tension typically reveal a longer latency for action of 20-HETE compared with PE, prompting us to examine responses over a longer time course. Indeed, when we treated cells with 20-HETE for prolonged periods, a low, sustained rise of [Ca\(^{2+}\)]\(_i\) was observed (Fig. 10B). A similar plateau was observed with PE (Fig. 10B, right).

Recent evidence indicates that Rho-kinase pathways are essential for NFAT activation in B cells (8, 17). This led us to examine whether NFAT translocation in pulmonary artery cells might also involve Rho-kinase. Smooth muscle cells were pretreated with the Rho-kinase inhibitor Y-27632 for 30 min and then stimulated with PE, 20-HETE, or vehicle for a further 30 min. Y-27632 inhibited PE- and 20-HETE-induced NFAT nuclear translocation in a concentration-dependent manner (Fig. 11), supporting the involvement of Rho-kinase in con-
strictor-induced NFAT translocation in pulmonary artery smooth muscle cells.

**DISCUSSION**

We investigated the regulation of the key transcription factor NFAT in rat and human pulmonary artery smooth muscle. The constrictors PE and 20-HETE induced comparable nuclear translocation of NFAT when studied both in vitro and in situ, with maximal nuclear localization induced at 30 min. The translocation was inhibited by the calcineurin inhibitors CsA and FK-506. Western blot analysis of cell lysates revealed a 140-kDa band for NFAT protein, corresponding to NFAT3, and constrictors caused translocation from the cytosol to the nucleus, an effect blocked by CsA. Whereas PE caused a rapid and transient increase in $[Ca^{2+}]$, 20-HETE elicited only persistent, low rise of $[Ca^{2+}]$. To our knowledge this is the first

Fig. 6. Constrictor-induced translocation of NFAT3 in rat and human pulmonary artery smooth muscle cells. Representative images show PE-induced translocation of NFAT3 from the cytosol to the nuclei of pulmonary artery smooth muscle cells, which was inhibited by pretreatment with CsA. In rat ($n = 3$) and human pulmonary artery ($n = 2$) smooth muscle cell cultures, pretreatment for 30 min with CsA inhibited both PE- and 20-HETE-induced NFAT3 nuclear translocation, indicating the involvement of calcineurin (bar graphs at right). ANOVA followed by Bonferroni, *$P < 0.01$ compared with corresponding control, #*$P < 0.01$ compared with corresponding constrictor.
demonstration of constrictor-induced NFAT activation in pulmonary artery smooth muscle cells. The involvement of Rho-kinase in nuclear translocation suggests a level of control of NFAT not previously recognized in smooth muscle.

Striking differences in NFAT isoform expression are becoming apparent from studies of smooth muscle from different vascular beds. Thoracic aortic smooth muscle expresses NFAT1, NFAT2, and NFAT4 (4, 39, 45), whereas cerebral artery expresses mainly NFAT4 and to a lesser extent NFAT3 (13, 14). Our studies of pulmonary artery smooth muscle show that NFAT3 was the only isoform observed and was subject to physiological regulation. Isoform-specific expression and regulation has been suggested to be an important question surrounding the roles of NFAT in the vasculature (19), so our studies provide valuable new information supporting the diversity in NFAT expression.

Earlier studies have suggested diversity in the actions of different agonists on NFAT activation. For example, endothe-
lin-1 and UTP cause more marked accumulation of NFAT4 in the nuclei compared with angiotensin II, EGF, or PGF2α in cerebral arteries (13). Moreover, some variability in time course is due to factors regulating the export of NFAT from the nucleus. A recent study reported that JNK2 phosphorylation hastens NFAT export, which could contribute to the pattern of NFAT residency in the nucleus (12). Our studies of two constrictors, PE and 20-HETE revealed essentially the same

Fig. 8. NFAT localization in smooth muscle of rat intralobar pulmonary artery at higher magnification. A: in control, vessels staining for NFAT (red) is diffuse in the cytosol (left). Nuclei were stained with SYTO-13 (green, middle) with little localization of NFAT in the nuclei, as demonstrated in the overlapped image at right and in the intensity plot (B) of the region highlighted by the arrow. C: representative confocal images of pulmonary artery smooth muscle from a PE-treated artery. The staining for NFAT (red) is overlapped with the nuclear stain SYTO-13 showing yellow stained nuclei and indicating localization of NFAT in the nuclei. This is demonstrated in the overlapped image at right and in the intensity plot (D) of the region highlighted by an arrow in C. The scale bars represent 50 μm.

Fig. 9. Nuclear localization of NFAT in intact rat intralobar pulmonary artery is dependent on calcineurin. Four separate arterial segments are shown, each treated as indicated at left. NFAT staining is shown in red in the left column, the nuclear stain SYTO-13 in green in the middle column, and images are overlapped at right. Yellow stained nuclei indicated nuclear localization of NFAT. Both PE (10 μM) and 20-HETE (156 nM) induced nuclear translocation of NFAT3 as illustrated with the NFAT3 antibody (top panels) and the pan antibody (bottom panels). In arteries pretreated with CsA (1 μM), NFAT3 staining was diffuse and most nuclei showed only green fluorescence staining with SYTO-13, indicating little or no translocation of NFAT3. Results are representative of 4 separate experiments using the pan antibody and 3 separate experiments using the NFAT3 antibody. Thus studies of intact tissues validate the cellular localization studies presented above. The scale bars represent 50 μm.
potency and pattern of activation of NFAT. The time course of activation and recovery was similar, with peak levels of nuclear translocation observed at 30 min. There was a suggestion of a biphasic response, especially for the human cells, although the early time points were not significantly different from control levels.

Moreover, NFAT translocation showed similar sensitivity to both CsA and FK-506, implicating the Ca\(^{2+}\)-sensitive phosphatase calcineurin. PE caused a large, transient rise of [Ca\(^{2+}\)]\(_i\), with short latency, which is typical for GPCR-mediated responses in smooth muscle but contrasts with the lower amplitude and slower responses to growth factors (27). We show here that 20-HETE caused a prolonged but low-amplitude rise of [Ca\(^{2+}\)]\(_i\). However, despite the different patterns of [Ca\(^{2+}\)]\(_i\) changes, the two constrictors showed similar time-course and pharmacological sensitivity of NFAT translocation. These data suggest that the initial rise of [Ca\(^{2+}\)]\(_i\) does not contribute to NFAT activation in this smooth muscle. This may not be surprising given the findings that NFAT activation is optimized by low, sustained changes of [Ca\(^{2+}\)]\(_i\) (7).

20-HETE is an arachidonic acid metabolite that is formed endogenously in the vasculature of the lung (25, 44). Formation of 20-HETE in smooth muscle is stimulated by many constrictor agonists, and it is suggested to be an important regulator of vascular tone (1, 6, 18). Addition of exogenous 20-HETE causes constriction of arteries of many vascular beds, including cerebral and renal arteries. 20-HETE does cause constriction of the intralobar pulmonary arteries in a concentration-dependent manner, and is \(1,000\)-fold more potent than PE (44). On the basis of this background, we examined the effects of 20-HETE at a concentration of 156 nM, a concentration known to cause close to maximal contraction in pulmonary arteries in vitro. However, when monitoring the rise of [Ca\(^{2+}\)]\(_i\), we found apparent differences in the responses to 20-HETE and PE. The small amplitude of the response to 20-HETE that we found could explain how this constrictor could cause an increase of tone in coronary arteries accompanied with negligible change of [Ca\(^{2+}\)]\(_i\) (31). 20-HETE is reported to enhance voltage-dependent Ca\(^{2+}\) current in...
cerebral artery smooth muscle (11). It remains to be established if influx contributes to the rise of \([\text{Ca}^{2+}]\), we observed. This is important to resolve, as NFAT activation is influenced by the source of \([\text{Ca}^{2+}]\) underlying the rise, with different responses to influx or release from intracellular stores (13).

Rho-kinase has recently been demonstrated to serve an essential role in activation of NFAT in immune cells, as assessed by genetic knockdown approaches (8, 17, 38). This revelation prompted us to examine the effect of Rho-kinase blockers in pulmonary artery smooth muscle, where we found the blocker Y-27632 significantly attenuated NFAT nuclear localization. This blockade was observed with both PE and 20-HETE, suggesting that Rho-kinase acts downstream of both constrictors. This is a novel observation for NFAT regulation involving 20-HETE, supporting the hypothesis that Rho-kinase is a key regulator of NFAT activation in smooth muscle cells.

NFAT is known to play key roles in cardiac growth and development, and in pathological hypertrophy (5, 9). A similar critical role has been identified in smooth muscle for patterning of the developing vasculature (15). Although relatively little is known of the genes regulated by NFAT in smooth muscle, a recent elegant study demonstrating that NFAT activation leads to decreased expression of the K\(_{\text{v2.1}}\) channel, Kv2.1 (2). Indeed, alterations in K\(_{\text{v}}\) channels have been reported in lung injury (43). This could account for continuous regulation of smooth muscle function in lung injury or inflammation (44). NFAT is also known to mediate growth factor and thrombin-induced vascular smooth muscle motility (28), with possible implications to hypertrophy in disease states, as is known to occur in pulmonary artery hypertension (21).

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REFERENCES

8. Foucault I, Le Bras S, Charvet C, Moon C, Altman A, and Deckert M. The adaptor protein 3BP2 associates with VAV guanine nucleotide exo...

30. Parmentier JH, Muthalif MM, Saeed AE, and Malik KU. Phospholipase D activation by norepinephrine is mediated by 12s-, 15s-, and 20-hydroxyeicosatetraenoic acids generated by stimulation of cytosolic...


