Intraluminal Pressure Is a Stimulus for NFATc3 Nuclear Accumulation

ROLE OF CALCIUM, ENDOTHELIUM-DERIVED NITRIC OXIDE, AND cGMP-DEPENDENT PROTEIN KINASE

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The transcription factor NFAT (nuclear factor of activated T-cells) is implicated in cardiac hypertrophy and vasculogenesis. NFAT activation, reflecting dephosphorylation by the calcium-dependent phosphatase, calcineurin, and subsequent nuclear localization, is generally thought to require a sustained increase in intracellular calcium. However, in smooth muscle we have found that elevation of calcium by membrane depolarization fails to induce an increase in nuclear localization of the NFATc3 isoform. Here, we demonstrate that physiological intravascular pressure (100 mm Hg) induces an increase in NFATc3 nuclear localization in mouse cerebral arteries. Pressure-induced NFATc3 nuclear accumulation is abrogated by endothelial denudation and by nitric-oxide synthase, cGMP-dependent kinase (PKG), and voltage-dependent calcium channels inhibition. We further show that exogenous nitric oxide, in combination with an elevation in calcium, is an effective stimulus for NFATc3 nuclear accumulation. c-Jun terminal kinase 2 (JNK) activity, which has been shown to regulate NFATc3 nuclear export, is also reduced by pressure, an effect that is prevented by pretreatment with a PKG inhibitor. Consistent with this, pressure-induced NFATc3 nuclear accumulation is independent of PKG in arteries from JNK2−/− mice. Collectively, our results indicate that both activation of the NO/PKG pathway and elevation of smooth muscle calcium are required for NFATc3 nuclear accumulation and that PKG inhibits JNK2 to decrease NFAT nuclear export. Our findings suggest that at physiological intravascular pressures NFATc3 is localized to the nucleus in smooth muscle cells of intact arteries and indicate a novel and unexpected role for nitric oxide/PKG in NFAT activation.

Pressure-induced mechanical deformation of arterial smooth muscle cells initiates a signaling cascade that leads to constriction (myogenic tone) (1). This serves to maintain tissue perfusion despite increases in blood pressure. In small intact cerebral arteries one aspect of this cascade is a graded membrane potential depolarization, which opens voltage-dependent calcium (Ca^{2+}) channels (VDCCs) and provides the increase in arterial smooth muscle intracellular Ca^{2+} concentration ([Ca^{2+}]_i) required for vasoconstriction (1–5). Intracellular Ca^{2+} in pressurized arteries not only regulates the contractile state of the smooth muscle cells but may also determine the activity of a variety of Ca^{2+}-dependent transcription factors, including cyclic AMP response element binding protein and NFAT (nuclear factor of activated T cells). Despite the importance of pressure in arterial function and the potential significance of Ca^{2+}-regulated transcriptional activity in smooth muscle, little is known about the relationship between pressure and Ca^{2+}-dependent transcription factor activation.

The NFAT transcription factor family is comprised of four well characterized members, designated NFATc1 (NFAT2/c), NFATc2 (NFAT1/p), NFATc3 (NFAT4/x), and NFATc4 (NFAT3). First identified as a Ca^{2+}-dependent transcription factor responsible for mediating coordinated changes in gene expression associated with T-cell activation (6–8), NFAT has since been shown to influence the phenotype of a diverse array of cell types outside of the immune system. NFAT has been shown to play a role in adipogenesis (9) and may be involved in osteoclastogenesis (10). In skeletal muscle NFAT is involved in development (11), growth (12), fast-twitch to slow-twitch iso-type switching (13), and hypertrophy (14). NFAT is also important in the cardiovascular system, where it regulates heart valve formation (15) and contributes to the pathogenesis of cardiac hypertrophy (16–18). Less is known about the role of NFAT in vascular smooth muscle, but recent reports have shown that NFAT is critical for embryonic vasculogenesis (19) and may play a role in regulating the phenotype of cultured vascular smooth muscle cells (20).

NFAT activation is regulated primarily through control of its subcellular localization. In general, a sustained or chronically oscillating increase in [Ca^{2+}]_i is thought to be necessary for sustained nuclear NFAT accumulation and NFAT-mediated

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The abbreviations used are: VDCC, voltage-dependent calcium (Ca^{2+}) channel; NFAT, nuclear factor of activated T-cells; [Ca^{2+}]_i, intracellular calcium concentration; HK, high potassium; JNK, c-Jun terminal kinase; P-JNK, phosphorylated JNK; PKG, cGMP-dependent protein kinase; PPKG, PKG inhibitor; PSS, physiological saline solution; WT, wild type; i-NNA, N-o-nitro-l-arginine; Indo, indomethacin; MAPK, mitogen-activated protein kinase.
transcription, although the recently reported effectiveness of transient elevations of Ca\textsuperscript{2+} in hippocampal neurons challenges the universality of this requirement (21). Elevation of global Ca\textsuperscript{2+} produced by a variety of mechanisms activates the Ca\textsuperscript{2+}-calmodulin-dependent protein phosphatase, calcineurin, which dephosphorylates the NFAT molecule at specific N-terminal serine residues. This induces a conformational change that exposes nuclear localization signals (22), allowing NFAT nuclear import. NFAT nuclear accumulation is subject to further regulation by serine/threonine kinases, which promote the export of nuclear NFAT. For example, nuclear c-Jun terminal kinase 2 (JNK2) has been shown to selectively promote the nuclear export of the NFATc3 isoform in both transfection studies (23) and native isolated cerebral arteries (24).

Recently, we have shown that NFATc3 is expressed in cerebral artery smooth muscle and can be induced to accumulate in the nucleus by uridine triphosphate (UTP) and other Go/11-coupled receptor agonists. This induction is mediated by calcineurin and is dependent on both sarcoplasmic reticulum Ca\textsuperscript{2+} release through inositol trisphosphate receptors and extracellular Ca\textsuperscript{2+} influx through VDCC (25). Unexpectedly, we found that opening of VDCC by membrane depolarization with 60 mM K\textsuperscript{+} (HK) fails to induce NFATc3 nuclear accumulation in this tissue (25). A summary of effects of pressure and a Ca\textsuperscript{2+} channel inhibition on NFATc3 nuclear translocation. Arteries were subjected to intraluminal pressures of 10 or 100 mm Hg for 30 min. Nisoldipine (1 \( \mu \)M) was applied for 30 min to arteries after stable constriction to 100 mm Hg. Data shown are the mean ± S.E. of 4–12 arteries per treatment (*, \( p < 0.0001 \) versus 10 mm Hg; #, \( p < 0.001 \) versus 100 mm Hg). B, representative images showing nuclear staining (green), NFATc3 staining (red), and nuclear localization of NFATc3 (white) in pressurized cerebral arteries. The scale bar represents 50 \( \mu \)m. SMC, smooth muscle.

Intraluminal Pressure Induces NFATc3 Activation

Nitric oxide and cGMP-dependent kinase (PKG) have been traditionally viewed as mediators of vasodilation through effects on large conductance, Ca\textsuperscript{2+}-activated potassium (BK) channels (26), VDCC (27, 28), phospholamban (29), and IRAG inositol trisphosphate receptor-associated cGMP kinase substrate, as well as by decreasing the Ca\textsuperscript{2+} sensitivity of the contractile process (30–32). Recent evidence indicates that NO/PKG can promote or inhibit vascular remodeling (33). PKG also has a significant role in the determination of the phenotype, contractile or synthetic, of vascular smooth muscle (34). Furthermore, studies from PKGi knockout mice provide evidence that PKG has an essential role in neovascularization (35). As indicated, NFAT also has an important role in vasculogenesis (19). However, the transcription factors affected by PKG have remained obscure.

In the present study we sought to establish the potential activation status of NFATc3 in intact cerebral arteries under conditions of physiological intravascular pressure by examining NFATc3 subcellular distribution at 100 mm Hg. At this pressure smooth muscle membrane potential is depolarized, intracellular Ca\textsuperscript{2+} is elevated, and the arteries are partially constricted. Based on the lack of effect of K\textsuperscript{+}-mediated depolarization on NFATc3 nuclear accumulation, we anticipated that NFATc3 would be primarily cytoplasmic in response to a pressure stimulus. Contrary to expectation, we found that NFATc3 accumulates in the nucleus when intraluminal pressure is elevated from 10 to 100 mm Hg. This pressure-induced
NFATc3 nuclear accumulation is dependent on Ca\(^{2+}\) influx through VDCC and is further dependent on endothelial-derived nitric oxide (NO) and PKG activity. Finally, we provide evidence that PKG acts to facilitate NFATc3 nuclear accumulation through inhibition of JNK2-mediated nuclear export. These results support the concept that the degree of NFATc3 activation reflects both physiological fluctuations of intracellular Ca\(^{2+}\) and endothelium-derived NO release and PKG activity.

**Experimental Procedures**

**Animals**—Adult male JNK2\(^{-/-}\) and C57BL/6 wild-type mice (20–25 g) were used. JNK2\(^{-/-}\) mice were kindly provided by Dr. Mercedes Rincon and have been previously described (36). C57B6 mice were obtained from Charles River Laboratories. All experiments involving animals were conducted in accordance with the guidelines for the care and use of laboratory animals (NIH publication 85-23, 1985) following a protocol approved by the Institutional Animal Use and Care Committee of the University of Vermont.

**Isolated Arterial Preparation**—Animals were euthanized with an overdose of pentobarbital solution (200 mg/kg intraperitoneally). After euthanasia the brain was removed and placed into oxygenated ice-cold physiological saline solution (PSS, for composition see “Chemicals and Buffers”). Posterior, cerebellar, and middle cerebral arteries were isolated and dissected from the surrounding connective tissue. Arteries were then cannulated and mounted in a specially designed, close-working distance arteriograph. All experiments with
intact arteries were done under continuous superfusion with PSS at 37°C. Only arteries that constricted in response to increased pressure were used. Endothelial denudation of cannulated vessels was accomplished by passage of 1 ml of air followed by 1 ml of PSS through the lumen. Arterial diameters were measured with a video dimension analyzer (Ionoptix).

Arterial Wall [Ca²⁺]—Cannulated arteries were loaded with the ratiometric Ca²⁺-sensitive dye fura-2 acetoxymethyl ester (Fura-2 AM, Molecular Probes) (2×10⁻⁶ M). Arterial [Ca²⁺] was calculated in Fura-2-loaded arteries as described previously (4, 37).

Immunofluorescence—At the end of each experiment cannulated arteries were fixed in the arteriograph with 4% formaldehyde in PSS (pH 7.4). After permeabilization and blocking of unspecific binding sites, primary antibody (rabbit anti-NFATc3 (Santa Cruz Biotechnology) or rabbit anti-P-JNK 1/2 (BIOSOURCE)) diluted 1:250 in 2% bovine serum albumin, phosphate-buffered saline was applied overnight at 4°C. Secondary antibody (Cy5-anti-rabbit IgG, Jackson ImmunoResearch Laboratories; 1:500 dilution) was applied for 1 h at room temperature. Nuclei were stained with the fluorescent nucleic acid dye SYTOX (Molecular Probes; 1:5000 dilution). Individual arteries were then mounted (Aqua Polymount mounting medium, Polysciences) onto glass slides and examined at 40× magnification using a Bio-Rad 1000 laser-scanning confocal microscope. NFATc3 or P-JNK was detected by monitoring the Cy5 fluorescence using an excitation wavelength of 650 nm and an emission wavelength of 670 nm. Specificity of immune staining was confirmed by the absence of fluorescence in arteries incubated with primary or secondary antibodies alone. For scoring of NFATc3-positive nuclei, multiple fields for each vessel were imaged and counted by two independent observers under double-blind conditions using Metamorph software (Universal Imaging Corp.). The software was programmed so that individual pixels within a given image would appear white if co-localization of the green nucleic acid stain and the Cy5-NFATc3 stain occurred. Thus, for quantification purposes, a cell was considered positive if co-localization (white) was uniformly distributed in the nucleus, whereas a cell was considered negative if no co-localization (green only) was observed. For P-JNK quantification, Metamorph software was used to determine the intensity of the antibody staining. For each vessel image log integrated intensity was measured in 5 representative regions (40×40 pixels) and averaged.

Chemicals and Buffers—All drugs and chemical reagents were purchased from Sigma unless otherwise specified. PSS contained 118.5 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl₂, 1.18 mM KH₂PO₄, 0.026 mM EDTA, 24 mM NaHCO₃, 11 mM glucose, 2.5 mM CaCl₂ (pH 7.4). This solution was continuously bubbled with 95% O₂, 5% CO₂ and heated to 37°C. High external K⁺ (60 mM) solution was made by iso-osmotic substitution of NaCl with KCl in the PSS. Drugs were added to the superfusate.

Statistics—Results are expressed as the means ± S.E. Statistical significance was tested at the 95% (p < 0.05) confidence level using one-way analysis of variance followed by Tukey’s multiple comparison test where applicable.

RESULTS

Elevation of Intraluminal Pressure Induces NFATc3 Nuclear Accumulation; Dependence on Ca²⁺ Influx through VDCC—To address the role of pressure as a stimulus for NFATc3 nuclear accumulation we used immunofluorescence microscopy to determine NFATc3 localization in arterial smooth muscle after...
elevation of intraluminal pressure from 10 to 100 mm Hg. This intraluminal pressure (100 mm Hg) is ~80% of the mean arterial blood pressure of these animals (111 ± 6 mm Hg, n = 6) and is close to the estimated physiological intraluminal pressure experienced by the cerebral vasculature (38). In cannulated cerebral artery segments from wild-type mice (WT) elevating intravascular pressure from 10 to 100 mm Hg increases smooth muscle [Ca^{2+}], from 133 ± 4 to 279 ± 53 nm, consistent with our previous results (3, 4) and those of others (2). Given the similar underlying mechanism of action, we anticipated that pressure, as with HK (25, 39), might not induce NFATc3 nuclear accumulation in arterial smooth muscle cells. Contrary to expectation, we found that an elevation in intravascular pressure from 10 to 100 mm Hg for 30 min induces NFATc3 nuclear accumulation (Fig. 1A and B). Inhibition of VDCCs with a dihydropyridine, nisoldipine (1 μM), dilated pressurized cerebral arteries (Fig. 2B) and prevented NFATc3 nuclear accumulation (Fig. LA). These results indicate that pressure induces NFATc3 nuclear accumulation, which is dependent on Ca^{2+} entry through VDCC.

Pressure-induced NFATc3 Nuclear Accumulation Is Dependent on Endothelium-derived NO—The endothelium plays an important role in regulating smooth muscle function through release of a variety of substances that modulate smooth muscle tone, including NO, prostanoids, and vasoactive peptides (e.g., endothelin). To determine whether the endothelium plays a role in pressure-induced NFATc3 nuclear accumulation, NFATc3 nuclear accumulation was evaluated in endothelium-denuded cerebral artery segments. Under these conditions increasing pressure to 100 mm Hg caused vasoconstriction but did not induce NFATc3 nuclear accumulation (Figs. 2, A and B), indicating that endothelium-derived factors are indeed involved in regulating smooth muscle NFATc3 nuclear accumulation.

To explore possible roles for endothelium-derived NO or prostanoids in NFAT activation, arteries were pretreated with the nitric-oxide synthase inhibitor, Nω-nitro-l-arginine (200 μM L-NNA), and/or the cyclooxygenase inhibitor, indomethacin (10 μM Indo) for 30 min. Combined treatment with Indo and L-NNA inhibited pressure-induced NFATc3 nuclear translocation in endothelium intact arteries, as did treatment with L-NNA alone (Fig. 2A). Indo alone, however, had no effect. These results indicate that endothelium-derived NO is required for pressure-induced NFATc3 nuclear accumulation, whereas prostanoids are not.

NO affects smooth muscle relaxation and phenotype by stimulating soluble guanylyl cyclase to produce cGMP, which in turn activates PKG (40). To determine whether the effects of NO on pressure-induced NFATc3 nuclear accumulation that we observed occurs through PKG activation, we utilized the specific PKG inhibitor, Rp-8-Br-PET-cGMPs (15 min of pretreatment with 10 μM). PKG inhibition completely prevented NFATc3 nuclear accumulation in arteries subjected to 100 mm Hg (Fig. 2A). These results indicate that endothelium-derived NO, acting through PKG, plays a major role in NFATc3 nuclear translocation mechanism in response to a pressure stimulus.

Cyclooxygenase, nitric-oxide synthase, or PKG inhibition or endothelial denudation potentiate pressure-induced vasoconstriction (Figs. 2, B and C). The fact that treatments that inhibit pressure-induced NFATc3 nuclear accumulation may promote vasorelaxation (i.e., nisoldipine) or vasoconstriction (i.e., L-NNA and Rp-8-Br-PET-cGMPs) indicates that the contractile state of the artery per se is not a determinant of NFATc3 nuclear accumulation.

NO Converts Depolarizing K^+ into an Effective Stimulus for NFATc3 Nuclear Accumulation—Although membrane potential depolarization with HK elevates intracellular Ca^{2+}, it is not a sufficient stimulus to induce significant NFATc3 nuclear accumulation in the smooth muscle cells of non-pressurized arteries. If elevated smooth muscle [Ca^{2+}], acts in concert with endothelial-derived NO to regulate NFATc3 nuclear localization, as suggested by the results above, the failure of HK to induce NFATc3 nuclear accumulation may reflect insufficient NO release. To test this possibility, the effect of membrane potential depolarization with HK in combination with an NO donor (1 μM NONOate for 30 min) was examined. HK plus NONOate promoted NFATc3 nuclear accumulation in the absence of a pressure stimulus (Fig. 3A), whereas HK alone or NONOate alone were not effective stimuli for NFATc3 activation. This effect is not related to changes in [Ca^{2+}], levels since NONOate addition partially dilates the arteries but does not modify HK-increased intracellular Ca^{2+} concentrations (Figs. 3, B and C) (41, 42). These results further support the idea that both an elevation in [Ca^{2+}], and NO are required for NFATc3 nuclear accumulation in vascular smooth muscle.
Nitric Oxide Decreases JNK2 Activity through PKG Activation—Although the Ca\(^{2+}\)/calmodulin/calcineurin-dependent control mechanism for NFAT nuclear localization has been clearly established, a role for PKG in the regulation of NFATc3 nuclear accumulation has not been reported. Using JNK2\(^{-/-}\) animals we have found that JNK2 plays an important role in promoting NFATc3 nuclear export in arterial smooth muscle (24), a result that supports previous reports of JNK2-mediated export of exogenously expressed NFATc3 in cultured cells (23). To address the possibility that JNK2 may be a downstream target of PKG, the effect of pressure on nuclear NFATc3 accumulation was examined in JNK2\(^{-/-}\) arteries in the presence or absence of PKG inhibitors. In arteries from JNK2\(^{-/-}\) mice elevated intraluminal pressure induces a Ca\(^{2+}\)-dependent (i.e. nisoldipine-sensitive) increase in nuclear NFATc3 that is comparable with that obtained in WT animals. However, in contrast to results obtained in WT mice, pressure-induced nuclear NFATc3 accumulation in JNK2\(^{-/-}\) mice is not dependent on PKG, as shown by the lack of an effect of PKG inhibition (Fig. 4A). These results indicate that JNK2 likely acts downstream of PKG and suggests that NO acts through PKG to decrease JNK2 activity, thereby increasing NFATc3 nuclear accumulation.

To further explore this mechanism, the effects of pressure on JNK activity were determined by immunofluorescence using an antibody that specifically recognizes the phosphorylated form of JNK1/2 (P-JNK). It is known that JNK is active when phosphorylated, and P-JNK levels reflect the degree of activity of the kinase. In arteries from WT mice, smooth muscle P-JNK levels are diminished by increasing pressure from 10 to 100 mm Hg, consistent with an inhibitory effect of PKG on JNK2 activity. In arteries pretreated with a PKG inhibitor, the levels of P-JNK are restored to resting levels (10 mm Hg) (Fig. 4B). Taken together these results provide evidence for a novel mechanism that links the NO/PKG pathway with JNK2 in regulating NFATc3 nuclear accumulation.

DISCUSSION
We provide the first evidence that intravascular pressure, which plays a central role in regulating vascular smooth muscle function, induces the accumulation of a Ca\(^{2+}\)-dependent transcription factor, NFATc3, into the nucleus of smooth muscle cells. Pressure-induced nuclear NFATc3 accumulation depends on VDCC-mediated Ca\(^{2+}\) entry and endothelium-derived NO. Nitric oxide acts through PKG, which in turn inhibits JNK2 activity. We propose that the PKG-mediated inhibition of JNK2 activity leads to a decrease in NFATc3 export (24), which in concert with Ca\(^{2+}\)-driven elevation in NFATc3 nuclear import, causes a significant NFATc3 nuclear accumulation at 100 mm Hg.

Pressure Induction of NFATc3 Nuclear Accumulation Requires Both an Elevation of Intracellular Ca\(^{2+}\) and Endothelium-derived NO—Smooth muscle cell intracellular Ca\(^{2+}\) levels at physiological pressure (100 mm Hg) are approximately 2–3-fold higher than those in low pressure (10 mm Hg) by virtue of pressure-induced membrane potential depolarization and VDCC activation (1–5). In native smooth muscle an elevation of intracellular Ca\(^{2+}\) by activation of VDCC is a necessary, but by itself insufficient stimulus for NFATc3 nuclear accumulation (25, 39). Here, we demonstrate that the endothelium-derived NO/PKG pathway acts as the second key stimulus (Figs. 2A and 3A). These results introduce a novel concept; stimuli that exert opposing influences on the contractile state of arterial smooth muscle, intracellular Ca\(^{2+}\) and NO, act cooperatively to facilitate activation of a transcription factor (NFATc3). Because NO/PKG can also regulate intracellular Ca\(^{2+}\) (26, 43, 44), this pathway may provide an additional level of regulation, acting as a negative feedback element to fine tune NFAT activation.

Novel Role of PKG; Transcription Factor Activation—PKG plays a prominent role in smooth muscle relaxation (45).
Emerging evidence indicates that PKG also has roles in vascular remodeling, phenotype determination, and vascular regeneration (33, 35, 47). Despite the involvement of PKG in the regulation of smooth muscle gene expression, regulation of transcription factors by PKG in smooth muscle is poorly understood. For example, PKG increases transcriptional activity of cyclic AMP response element–binding protein, ATF-1 (activation factor 1), TFI-II, and AP-1 (activator protein-1) in different cultured cells (48–52) and decreases SRP (serum response factor)-dependent transcription in cultured vascular smooth cells and cardiomyocytes (53). Here, we provide the first evidence that PKG, in concert with an elevation of intracellular Ca2+, promotes the activation of a smooth muscle transcription factor, NFAT.

NFAT activation is a dynamic process that depends on an increase in intracellular Ca2+ and subsequent calcineurin activation to overcome basal levels of nuclear kinase activity that oppose NFAT nuclear export (24). In principle, NO/PKG could contribute to NFAT3 nuclear accumulation by increasing its import or by decreasing its export. However, NO/PKG activation leads to a reduction of intracellular Ca2+ through activation of BK channels (26, 43, 45) and/or inhibition of L-type Ca2+ channels (54). If anything, this would be predicted to inhibit NFAT nuclear import, and PKG has in fact been shown to decrease NFAT activity in cultured cardiac myocytes through NFAT nuclear import, and PKG has in fact been shown to decrease NFAT activity in cultured cardiac myocytes through such a reduction of intracellular Ca2+ (54). In addition, there are no reports suggesting that calcineurin is directly activated by PKG. Thus, the NO/PKG pathway is likely to act instead by decreasing NFAT nuclear export.

JNK2 has been implicated in NFAT3 nuclear export in both transfection studies (23) and native isolated cerebral arteries (24). In JNK2 knockout (−/−) mice elevation of intracellular Ca2+ alone is a sufficient signal to stimulate nuclear accumulation of NFAT3, because the nuclear export rate of NFAT3 is slowed in these animals (24). Using the JNK2 knockout (−/−) mouse model we tested the hypothesis that NO/PKG facilitates NFAT3 nuclear accumulation through inhibition of JNK2 activity and found that inhibition of PKG had no effect on NFAT3 nuclear accumulation in the absence of JNK2 (Fig. 4A). This is in sharp contrast to the effect of Rp-8-Br-PET-cGMPs-mediated PKG inhibition in arteries from WT mice, which prevents NFAT3 nuclear accumulation. The implication is that JNK2 activity is suppressed in pressurized arteries. This prediction is supported by experiments demonstrating that the levels of activated JNK (P-JNK) in smooth muscle are decreased in response to elevated intraluminal pressure, an effect prevented by PKG inhibition (Fig. 4B). Taken together, these results suggest that NO/PKG exerts a negative regulatory influence on JNK2 activity. An additional implication of these results is that the production of NO by the endothelium may increase in response to elevations in pressure, consistent with recent results from rat mesentery and cerebral arteries (55–57).

The signal transduction steps between PKG and inhibition of JNK2 are not known. PKG has been reported to activate mitogen-activated protein kinase (MAPK) phosphatase, which suppresses the activity of the MAPK cascade (58, 59). Because JNK2 is a downstream target of the MAPK cascade, it is possible that PKG inactivates JNK2 through the activation of MAPK phosphatase.

Physiological Implications—NFAT is essential for embryonic vascular patterning and has been implicated in the development of cardiac hypertrophy (18, 19, 60, 61). PKG has also been reported to be involved in vascular regeneration (35). These observations, taken together with the results presented here, suggest the intriguing possibility that NFAT might provide a link between PKG and vasculogenesis. Recently, it has been reported that calcineurin and/or NFAT activity is necessary to maintain the differentiated state of cultured smooth muscle cells (20, 62). Interestingly, PKG has also been implicated in the maintenance of the vascular smooth muscle cell contractile phenotype. Lincoln et al. (46) show that expression of PKG disappears in smooth muscle cells that have adopted the synthetic phenotype; over-expression of PKG in these cells results in a restoration of a contractile-like phenotype. Our data are consistent with these findings and provide the first evidence that the NFAT3 subtype is activated in vascular smooth muscle under normal physiological pressures and may, thus, contribute to the maintenance of the contractile phenotype.

Our results suggest that a complex interplay of control mechanisms involving intracellular Ca2+ and endothelium NO acts to regulate NFAT3 activity under normal conditions. Vascular dysfunction is often characterized by changes in arterial smooth muscle Ca2+ and by alterations in endothelium function, raising the possibility that dysregulation of NFAT activation may contribute to vascular pathologies.

In conclusion, intraluminal pressure is a stimulus for NFAT3 nuclear translocation. Pressure-induced NFAT3 nuclear translocation requires Ca2+ influx through VDCC and PKG activation by endothelium-released NO with a concomitant decrease in smooth muscle JNK2 activity (see Fig. 5). These results provide the first evidence that endogenous nitric oxide and PKG are required for NFAT3 nuclear accumulation in smooth muscle, independent of nitric oxide effects on intracellular Ca2+, and suggest a key role of endothelium in the regulation of arterial smooth muscle transcriptional activity.

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