Upregulation of Intermediate-Conductance Ca\(^{2+}\)-Activated K\(^+\) Channels (KCNN4) in Porcine Coronary Smooth Muscle Requires NADPH Oxidase 5 (NOX5)

Hope K. A. Gole\(^1\), Darla L. Tharp\(^1\), Douglas K. Bowles\(^{1,2,3\ast}\)

\(^1\) Department of Biomedical Sciences, University of Missouri Columbia, Columbia, Missouri, United States of America, \(^2\) Dalton Cardiovascular Research Center, University of Missouri Columbia, Columbia, Missouri, United States of America, \(^3\) Medical Pharmacology and Physiology, University of Missouri Columbia, Columbia, Missouri, United States of America

Abstract

**Aims:** NADPH oxidase (NOX) is the primary source of reactive oxygen species (ROS) in vascular smooth muscle cells (SMC) and is proposed to play a key role in redox signaling involved in the pathogenesis of cardiovascular disease. Growth factors and cytokines stimulate coronary SMC (CSMC) phenotypic modulation, proliferation, and migration during atherosclerotic plaque development and restenosis. We previously demonstrated that increased expression and activity of intermediate-conductance Ca\(^{2+}\)-activated K\(^+\) channels (KCNN4) is necessary for CSMC phenotypic modulation and progression of stenotic lesions. Therefore, the purpose of this study was to determine whether NOX is required for KCNN4 upregulation induced by mitogenic growth factors.

**Methods and Results:** Dihydroethidium micro-fluorography in porcine CSMCs demonstrated that basic fibroblast growth factor (bFGF) increased superoxide production, which was blocked by the NOX inhibitor apocynin (Apo). Apo also blocked bFGF-induced increases in KCNN4 mRNA levels in both right coronary artery sections and CSMCs. Similarly, immunohistochemistry and whole cell voltage clamp showed bFGF-induced increases in CSMC KCNN4 protein expression and channel activity were abolished by Apo. Treatment with Apo also inhibited bFGF-induced increases in activator protein-1 promoter activity, as measured by luciferase activity assay. qRT-PCR demonstrated porcine coronary smooth muscle expression of NOX1, NOX2, NOX4, and NOX5 isoforms. Knockdown of NOX5 alone prevented both bFGF-induced upregulation of KCNN4 mRNA and CSMC migration.

**Conclusions:** Our findings provide novel evidence that NOX5-derived ROS increase functional expression of KCNN4 through activator protein-1, providing another potential link between NOX, CSMC phenotypic modulation, and atherosclerosis.

Citation: Gole HKA, Tharp DL, Bowles DK (2014) Upregulation of Intermediate-Conductance Ca\(^{2+}\)-Activated K\(^+\) Channels (KCNN4) in Porcine Coronary Smooth Muscle Requires NADPH Oxidase 5 (NOX5). PLoS ONE 9(8): e105337. doi:10.1371/journal.pone.0105337

Editor: David Jourd'heuil, Albany Medical College, United States of America

Received October 26, 2013; Accepted July 23, 2014; Published August 21, 2014

Copyright: © 2014 Gole et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the National Institutes of Health [HL52490, RR18276](http://www.nih.gov/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: BowlesD@missouri.edu

Introduction

One of the central components of cardiovascular disease (CVD) is atherosclerosis, which is a slow degenerative process characterized by remodeling of the arterial wall and formation of atherosclerotic plaques [1,2]. A key to plaque development during atherosclerosis is vascular smooth muscle cell (SMC) phenotypic modulation, proliferation, and migration into the neointimal region of the vessel [3,4,5]. The ability of vascular SMCs to undergo phenotypic modulation in response to physiological and pathophysiological cues is unique [6,7,8,9]. The transition from a differentiated to a de-differentiated state in response to vascular injury, is marked by a suppression of SMC differentiation genes and an increased autocrine/paracrine generation of basic fibroblast growth factor (bFGF), platelet derived growth factor-B (PDGF-BB), transforming growth factor (TGF-B), and angiogenesins II (AngII) [7,8,9,10,11,12].

We have previously shown that PDGF-BB induced coronary SMC (CSMC) phenotypic modulation requires the functional upregulation of intermediate-conductance Ca\(^{2+}\)-activated K\(^+\) channels (KCNN4) [7]. KCNN4 are voltage-independent channels composed of six membrane-spanning domains, modulated by intracellular Ca\(^{2+}\) to induce hyperpolarization [13]. Within the vasculature these channels regulate membrane potential and calcium signaling in addition to playing a role in vasorelaxation and neointimal formation associated with CVD [13,14,15,16]. Studies have shown that KCNN4 upregulation is required for mitogen-induced suppression of SMC markers as well as vascular SMC migration and proliferation, and has been shown to occur during atherosclerosis and restenosis indicating these channels play a key role in coronary plaque formation [7,15,17,18]. KCNN4 upregulation has previously been shown to occur via transcriptional activation of activator protein-1 (AP-1) [7,18,19] and reduction in repressor element-1 silencing transcription factor.
Isolation of coronary arteries

Yucatan swine obtained from the Sinclair Research Farm (Columbia, MO, USA) were anesthetized with telazol (5 mg/kg) and xylazine (2.25 mg/kg), followed by administration of heparin (1000 U/kg). Swine were euthanized by removal of the heart, which was immediately placed in 4°C physiological saline solution (PSS). The right coronary artery (RCA) was isolated, cleaned of fat and connective tissue, placed in low Ca²⁺ PSS and stored at 4°C until use (0–1 days).

Porcine CSMC culture

Primary CSMCs were isolated from the medial portion of a castrated male yucatan porcine RCA following removal of adventitia and mechanical denudation to remove endothelium, as previously described [7,54]. Cells were initially plated at 1.5 × 10⁴ cells/cm² in custom DMEM/F-12 media (GIBCO RR070050) without L-valine, containing pyridoxine HCl, 100 U/mL pen/strep, 1.6 mM L-glutamine, and 10% FBS for 4–6 days until post-confluent. Media was changed every two days. Subsequent passages were plated at the same density in DMEM/F-12 media (GIBCO 11320-033) containing 100 U/mL pen/strep, 1.6 mM L-glutamine, and 10% FBS. Cells (passage 2–6) were then serum starved 5 days to maximize smooth muscle differentiation marker gene [smooth muscle alpha-actin (SMAα), smooth muscle myosin heavy chain (SMMHC), and smoothelin (SE)] expression levels, as previously shown [7].

ROS detection

O₂⁻ anion production was determined using dihydroethidium (DHE) micro-fluorography. In the presence of the O₂⁻ anion, DHE (which is freely permeable to cells) is oxidized to 2-hydroxyethidium [55]. The 2-hydroxyethidium becomes trapped intracellularly, producing fluorescence at an excitation wavelength of 488–520 nm with an emission spectrum of 610 nm [56,57]. Unlike techniques utilizing lucigenin, DHE has little capacity for redox cycling thus the fluorescence detected correlates well to the level of cellular O₂⁻. Cultured CSMCs were grown to confluence on cover slips, serumstarved for 5 days, and treated with bFGF (25 ng/mL, Upstate), bFGF+apocynin (Apo 1.0 mM, Sigma), bFGF+Tempol (1.0 mM, Sigma), Tempol, or Apo for 24 hours. Apo is used as an NOX inhibitor, disrupting the assembly of cytosolic and membrane components necessary for enzymatic activity [44,47]. Under minimal light conditions, cells were incubated with DHE (10 μM, Sigma) for 30 minutes at 37°C, with the addition of the nuclear stain DAPI (10 mM, Molecular Probes) for the last 15 minutes. Following incubation, the cover slips were mounted on microscope slides for imaging. Images were obtained at the Molecular Cytology Core (University of Missouri, Columbia).

Quantitative reverse-transcriptase PCR (qRT-PCR)

qRT-PCR was performed as previously described [7,58,59]. Cells in TRIzol were quick frozen in liquid nitrogen, stored at −80°C until processed, and total RNA was isolated according to the published TRIzol protocol. cDNA was transcribed from total RNA using Applied Bioscience high-capacity cDNA reverse transcription kit and qRT-PCR was performed on a MyiQ iCycler (Bio-Rad, model 170-9770). Each 25 μL reaction contained 1X Syber Green Master Mix (Bio-Rad), 0.8 μM forward and reverse primers, and 1 μg of cDNA. Each reaction was initiated by a 95°C hold for 3 minutes in order to activate heat stable Taq polymerase, and reaction conditions were optimized for each set of primers: KCNN4, NOX1, NOX2, NOX4, NOX5.
Target gene expression was normalized to 18S ribosomal RNA using the $2^{-\Delta\Delta CT}$ method [60,61]. To ensure reliable comparison between target genes, PCR linearity and efficiency was verified by creating a standard curve plotting the critical threshold versus log of the dilution of cDNA. Each primer set had a slope of 3.3±0.3, indicating a 90–100% efficiency, and an $R^2$ value >0.99 providing strong confidence of correlating values.

Immunohistochemistry

Immunohistochemistry was performed as previously described [59]. RCA sections were incubated with avidin–biotin two-step blocking solution (Vector SP-2001) to inhibit background staining and 3% hydrogen peroxide to inhibit endogenous peroxidase. Non-serum protein block (Dako X909) was then applied to inhibit non-specific protein binding. Sections were incubated at 4°C overnight with primary antibodies KCNN4 (1:600, Chemicon), nitrotyrosine (1:400, Chemicon), or SMαA (1:200, DAKO). After appropriate washing steps, sections were incubated with biotinylated secondary antibody in phosphate-buffered saline containing 15 mM sodium azide and peroxidase-labeled streptavidin (Dako LSAB+ kit, peroxidase, K0690). Diaminobenzidine (DAB, Dako) was applied 5 minutes for visualization of the reaction product, sections were then counterstained with haematoxylin, dehydrated, and coverslipped. Images of the sections were obtained using an Olympus BX40 photomicroscope and Spot Insight Color camera (Diagnostic Instruments). The relative area and mean density of positive staining for KCNN4 were determined for each section of interest utilizing ImagePro Plus (Media Cybernetics).

Whole cell voltage clamp

Whole cell $K^+$ current ($I_K$) was measured as previously described [7,62,63]. Cultured CSMCs were trypsinized then suspended in a low-Ca$^{2+}$PSS containing 20 mM HEPES and stored at 4°C until use (0–1 days). Normal PSS containing (in mM) 2 CaCl$_2$, 10 glucose, 10 HEPES, 5 KCl, 1 MgCl$_2$, and 138 NaCl, pH 7.4 was used to superfuse the cells at room temperature under gravity flow. Pipettes (2–6 MΩ) were filled with solution containing (in mM) 0.50 CaCl$_2$ (0.5 μM free Ca$^{2+}$), 10 KCl, 10 NaCl, 1 MgCl$_2$, 10 HEPES, and 10 K$_2$EGTA, with pH 7.1. To confirm calcium-sensitivity, potassium currents were also measured under nominally calcium free internal (Cai) conditions using normal PSS containing (in mM) 0.1 CaCl$_2$, 10 glucose, 20 HEPES, 5 KCl, 1 MgCl$_2$, and 138 NaCl, pH 7.4 as the superfusate, and (in mM) 120 KCl, 10 NaCl, 1 MgCl$_2$, 10 HEPES, 10 K$_2$EGTA, pH 7.1 as the pipette solution. After seal formation (seal resistance $>$1 GΩ), series resistance was monitored for determination of sufficient whole cell access (series resistance below 25 MΩ). From a holding

Table 1. Primer Sequences (5’ to 3’).

| Target Gene | Forward Primer | Reverse Primer |
|-------------|----------------|----------------|
| KCNN4       | CCC ATC ACA TTC CTG ACC AT | GTC CTT CCT AGG GCT GTG TT |
| NOX1        | AAT GGC ATC CCT TTA CCC TGA CCT | CTT GGA ACT GGC GAA TTC GTG TGT |
| NOX2        | TAA GCA GTG CAT CTC CAA CTC CGA | GCC ATT ATC TGG GCA TTT GGC AGT |
| NOX4        | TGC ATA ACA AGT TTT GGC AAG A | ATC CCA TCT GTT TGA CTG AG |
| NOX5        | TGC TGA GAG ATT CTT CGC CCT CTT | AGG AAC TGG AGT TTG TCC GTG GGA |
| 18S         | CGG CTA CCA CAT CCA AGG AA | AGC TGG AAT TAC CGC GGC |

doi:10.1371/journal.pone.0105337.t001

Figure 1. bFGF increased NOX-derived $O_2^-$ production. bFGF (25 ng/mL) treatment for 24 hours increased DHE fluorescence (red) in CSMCs compared to control. Addition of the NOX inhibitor Apo (1.0 mM) and superoxide scavenger Tempol (1.0 mM) blocked bFGF induced $O_2^-$ production. DAPI stained nuclei are shown in blue. Data presented are representative images of three different experiments.

doi:10.1371/journal.pone.0105337.g001
potential of −80 mV, currents were elicited by 500-ms step depolarizations to potentials ranging from −70 mV to +100 mV (in 10 mV increments). Current was recorded in control cells, cells treated with bFGF (25 ng/mL), or cells treated with bFGF+Apo (1.0 nM) for 24 hours, both before and after addition of TRAM-34 (a selective KCNN4 channel blocker, 100 nM) to the superfusate. To account for differences in cell membrane surface area, current densities (pA/pF) were obtained for each cell by normalization of whole cell current to cell capacitance. An initial area, current densities (pA/pF) were obtained for each cell by

Table 2. NOX siRNA sequences (5′ to 3′).

| Target Gene | Sense   | Antisense           |
|-------------|---------|---------------------|
| NOX2        | GGA UGG AGG UGG GAC AUA Att | UAU UGU CCC ACC UCC AUC Ctg |
| NOX4        | GUG UCC UAC UGA AAC CAA Att | UUU GGU UUC AGU ACA Cat |

doi:10.1371/journal.pone.0105337.t002

Figure 2. Inhibition of NOX prevented bFGF upregulation of KCNN4 mRNA expression. KCNN4 mRNA expression increased approximately 2.5 fold in both RCA (n=4–6) and CSMCs (n=15–17) with bFGF (50 ng/mL and 25 ng/mL, respectively) treatment compared to control. Addition of Apo (1.0 mM) abolished bFGF-induced increases in KCNN4 expression, and alone also significantly decreased basal KCNN4 expression in CSMCs. *P<0.05 vs respective bFGF. #P< 0.05 vs respective bFGF.

doi:10.1371/journal.pone.0105337.g002

AP-1 Plasmid Transfection

4 day serum-starved cultured CSMCs were trypsinized, placed in basic smooth muscle nucleofection solution (Axama), and transected with pAP-1 Luc Cis-Reporter plasmid in which firefly luciferase gene expression in the reporter plasmid is controlled by a synthetic promoter containing direct repeats of the AP-1 transcription recognition sequence (1.0ug, Stratagene 219074). In addition to the desired plasmids, each sample contained 1 ×10^6 cells, 100uL of nucleofection solution and 0.05uL of Renilla luciferase. Samples were placed in electroporation cuvettes (Amaza) and nucleofected using program D-33 of the Amaza nucleofector device, as previously described [7]. Following nucleofection, cells were immediately placed in RPMI media, incubated at 37°C and 5% CO2 for 15 minutes, then plated in 6-well plates containing DMEM/F-12 media (GIBCO 11320-033) with 100 U/mL pen/strep, 1.6 mM L-glutamine, and 10% FBS. Upon adherence (~4 hours), cells were serum-starved for 4 hours then incubated with drug treatments for an additional 24 hours in serum free media. Following a 24-hour treatment period, the luciferase activity assay was performed.

AP-1 Luciferase Activity Assay

After treatment, nucleofected CSMCs were washed in PBS and lysed with luciferase assay cell lysis buffer (Stratagene). The cell lysate was mixed with an equal amount of luciferase assay reagent (Stratagene) and luciferase activity measured using a luminometer (Monolight 2010; Analytical Luminescence Laboratory, San-Diego, CA). Results were expressed as a ratio of the firefly luciferase to Renilla luciferase luminescence normalized to control cells (fold induction).

Adenovirus and shRNA

Replication-deficient adenovirus for GFP-tagged NOX5 shRNA (AdNOX5shRNA; Open Biosystems, GIPZ Human clone V3LHS_353966) was generated using AdEasy adenoviral vector systems (Agilent). CSMCs were serum-starved for 24 hours, and then infected with AdNox5shRNA at an MOI of 100 plaque forming units for 48 hours at 37°C. Cells were cultured in non-virus containing serum-free media for an additional 4 days, and then treated with bFGF (25 ng/mL) for 24 hours. Following treatment, cells were frozen in TRIzol and qRT-PCR was performed as described above.

Nucleofection and siRNA application

Prior to performing nucleofection with NOX isoform siRNA’s, time course experiments were conducted to determine the duration of gene knockdown for each isoform. 90–100% confluent, 4 day serum-starved CSMCs were trypsinized and placed in basic smooth muscle cell nucleofection solution (Amaza) with either a negative control siRNA, NOX2 siRNA, or NOX4 siRNA. Nucleofection was performed as described above. Each sample contained 1.0 ×10^6 cells, 100 uL of nucleofection solution, and 2.5 ng of siRNA. Following treatment, cells were frozen in TRIzol and qRT-PCR was performed as described above. The NOX isoform siRNA’s were custom designed by Ambion (Table 2) and the negative control siRNA was purchased from Ambion (Silencer Negative control siRNA #1).

Chemotaxis

Seven days after transduction with control adenovirus or AdNOX5shRNA, postconfluent porcine coronary SMCs were plated at 30,000–40,000 cells/well in the upper chamber of a 10-
μm-pore, 96-well chemotaxis chamber (Millipore). The following solutions were placed in the lower chamber (diluted in serum-free media): vehicle, PDGF-BB (30 ng/ml), and PDGF-BB + TRAM-34 (100 nM). The chamber was placed at 37°C for 4 h. Cells from the upper chamber were removed, and the filters were stained using Diff-Quik staining kit (Fisher Scientific). The migrated cells in a 20x field were manually counted.

Statistics
All data are presented as mean±SE. One-way ANOVA was used for all group comparisons, and significance was defined as P<0.05.

Results
bFGF increased NOX-derived O$_2^-$ production
To determine if bFGF upregulates NOX-derived O$_2^-$ production, cultured CSMCs were treated with bFGF, the NOX inhibitor Apo, and the O$_2^-$ scavenger Tempol. DHE microfluorography showed that bFGF increased O$_2^-$ production, Apo inhibited both bFGF induced and basal O$_2^-$ production, and Tempol inhibited bFGF induced O$_2^-$ production (Figure 1).

Inhibition of NOX prevented bFGF upregulation of KCNN4 mRNA expression
qRT-PCR was used to determine if NOX is required for bFGF-induced increases in KCNN4 mRNA expression. Cultured CSMCs were treated with bFGF, bFGF+Apo, or Apo for 24 hours. Freshly isolated RCA sections were mechanically denuded to remove the endothelium, placed in RPMI, treated with bFGF, bFGF+Apo, or Apo, and incubated at 37°C for 24 hours. In RCA sections, treatment with bFGF significantly increased KCNN4 expression, which was inhibited by treatment with Apo (Figure 2). In cultured CSMCs, treatment with bFGF significantly increased KCNN4 expression while treatment with Apo inhibited both bFGF increased and basal KCNN4 expression (Figure 2).

Inhibition of NOX prevented bFGF upregulation of KCNN4 protein expression
To determine if NOX-mediated, bFGF-induced increases in KCNN4 mRNA translate to increased protein expression, freshly isolated RCA sections were treated with bFGF, bFGF+Apo, or Apo for 24 hours, formalin fixed, and sectioned. Medial KCNN4 protein expression was increased in response to treatment with bFGF, while addition of Apo abolished the bFGF increase in KCNN4 protein expression (Figure 3A, 3C). Sections were also stained with the smooth muscle marker SMαA to confirm the media was primarily composed of CSMCs, and to confirm that the uneven KCNN4 staining pattern was not artifact. Interestingly,
A

- TRAM

Control

+ TRAM

bFGF

bFGF+Apo

bFGF-Ca^{2+}_i

B

TRAM-34 Sensitive Current (pA/pF)

Control  bFGF  bFGF+Apo  bFGF-Ca^{2+}_i

*
the staining pattern of the oxidative stress marker nitrotyrosine was similar to KCNN4, consistent with increased oxidative stress in regions of the media with elevated KCNN4 protein expression (Figure 3B).

Inhibition of NOX prevented bFGF upregulation of KCNN4 activity

Whole-cell voltage clamp was used to determine if NOX-mediated, bFGF-induced upregulation of KCNN4 results in increased KCNN4 channel activity. To isolate current through KCNN4, K⁺ current was measured before and after addition of the specific KCNN4 channel blocker TRAM-34. Widely used as an inhibitor of KCNN4, previous patch-clamp studies have shown that TRAM-34 has a 1,000 times greater selectivity for KCNN4 versus other calcium, potassium, and sodium channels [64], and previous specificity screens comparing 30 receptors and transporters have confirmed that it is a highly specific blocker of KCNN4 [17]. To confirm calcium sensitivity, TRAM-34 sensitive K⁺ current was measured following treatment with bFGF using a nominally calcium free pipette solution. Representative ensemble currents from individual cultured CSMCs (Figure 4A) and TRAM-34 sensitive currents (Figure 4B) demonstrate significantly increased KCNN4 current in response to treatment with bFGF. Consistent with KCNN4 mRNA and protein expression, treatment with Apo prevented bFGF-induced increases in KCNN4 channel activity, indicating that NOX-derived upregulation of KCNN4 results in functional KCNN4 channels at the membrane. Absence of TRAM-34 sensitive current in nominal internal calcium conditions confirmed measurement of calcium-sensitive potassium current (Figure 4).

NOX regulation of KCNN4 involves the AP-1 transcription factor

To determine if the AP-1 transcription factor is involved in NOX regulation of KCNN4, cultured CSMCs were nucleofected with pAP-1 Luc Cis-Reporter plasmid, and treated with bFGF, bFGF+Apo, or Apo for 24 hours. Following treatment, firefly luciferase activity was measured and normalized to Renilla luciferase activity. Analysis demonstrated a significant increase in AP-1 promoter reporter activity in response to bFGF, which was inhibited by treatment with Apo (Figure 5).

Porcine coronary smooth muscle expressed NOX1, NOX2, NOX4, and NOX5 isoforms

To determine which NOX isoforms are expressed in porcine coronary smooth muscle, freshly isolated RCA sections and cultured CSMCs were harvested as described above. qRT-PCR analysis showed that all four cardiovascular NOX isoforms (NOX1, NOX2, NOX4, and NOX5) were expressed in both RCA and CSMCs, with NOX4 having the highest mRNA expression (Figure 6).

Knockdown of NOX5 prevented bFGF upregulation of KCNN4 mRNA expression

To determine if NOX5 is required for NOX regulation of KCNN4, CSMCs were infected with adenovirus expressing shRNA targeted to knockdown NOX5 (AdNOX5shRNA), treated with bFGF for 24 hours, isolated, and prepped for qRT-PCR. Knockdown of NOX5 (Figure 7B) completely prevented bFGF-
induced increases in KCNN4 mRNA expression (Figure 7A). While AdNOX5shRNA reduced NOX5 mRNA expression by \(90\%\) (Figure 7B), it did not reduce mRNA expression of other NOX isoforms, such as NOX 1, 2 or 4 (0.68 \( \pm \) 0.27, 0.93 \( \pm \) 0.47, 0.83 \( \pm \) 0.07) versus respective controls (1 \( \pm \) 0.38, 1 \( \pm \) 0.4, 1 \( \pm \) 0.1). Furthermore, knockdown of neither NOX2 (Figure 8A) nor NOX4 (Figure 8B) prevented upregulation of KCNN4 mRNA indicating bFGF upregulation of KCNN4 was NOX5 specific.

Knockdown of NOX5 prevented coronary smooth muscle cell migration

To determine if NOX5 plays a role in migration, CSMCs were infected with adenovirus expressing shRNA targeted to knockdown NOX5 (AdNOX5shRNA), plated in a chemotaxis chamber, treated with the chemotactic agent PDGF-BB with or without TRAM-34 for 4 hours, stained, and counted (Figure 9). Compared to control adenovirus in the presence of PDGF-BB (36.50 \( \pm \) 2.11 cells/field, \( n = 8 \)), knockdown of NOX5 significantly inhibited migration (18.16 \( \pm \) 2.99 cells/field, \( n = 6 \)). Lack of further inhibition of migration by TRAM-34 in the NOX5 knockdown group (16.67 \( \pm \) 2.49 cells/field, \( n = 6 \)) supports a NOX5-dependent KCNN4 role in CSMC migration.

Discussion

Oxidative stress induced by ROS plays a key role in atherogenesis and atherosclerotic plaque instability [26, 49, 65, 66]. Increased production of ROS activates reduction-oxidation (redox)-sensitive signaling pathways resulting in endothelial activation, oxidative modification of lipids, proliferation of vascular SMCs, and recruitment of vascular SMCs into atherosclerotic plaques [4, 26, 49]. Studies have shown that the \( O_2^- \) anion is one of the most important ROS in the vasculature [67]. In vascular SMCs, \( O_2^- \) production is increased in response to numerous stimuli including growth factors [34, 38, 68]. Our results demonstrate that treatment with the angiogenic factor, bFGF, significantly increased CSMC \( O_2^- \) production. Previous studies have shown that within the vasculature, NOX is the primary source of ROS including \( O_2^- \) [44, 69, 70]. Consistent with these reports, treatment with the NOX inhibitor Apo abolished both bFGF-stimulated and basal \( O_2^- \) production in CSMCs (A, \( n = 5-9 \)). Similarly, nucleofection with siNOX4 significantly reduced NOX4 expression but did not prevent upregulation of KCNN4 mRNA expression by bFGF (25 ng/mL) in CSMCs (B, \( n = 5-9 \)). *\( P < 0.05 \) vs control. doi:10.1371/journal.pone.0105337.g008
activated by alternative peroxidases and MPO secreted by one cell type can be taken up and utilized by an alternative cell type [72].

In recent years, studies have shown that KCNN4 play a key role in the regulation of vascular SMC proliferation and migration associated with atherogenesis, stenosis, and plaque formation [7,15,17,18]. We were able to mimic previous reports of vascular SMC KCNN4 upregulation in response to growth factors [7,16,73], demonstrating increased KCNN4 mRNA expression, protein expression, and channel activity in response to treatment with bFGF. ROS and NOX are known to regulate ion channels, playing a key role in Ca$^{2+}$ signaling and Ca$^{2+}$ release from intracellular stores [40,74]. Inhibition of the bFGF-induced increase in KCNN4 mRNA, protein, and channel activity by treatment with Apo indicates a critical role for NOX in KCNN4 regulation and provides a novel pathway for NOX to contribute to the progression of atherosclerosis.

Multiple transcription factors including AP-1, NFkB, and c-Jun NH2-terminal kinase (JNK) are redox-sensitive and activated during atherosclerosis [70,75,76,77,78]. Interestingly, previous studies have shown that growth factor induced upregulation of KCNN4 occurs via transcriptional activation of AP-1 [7], indicating that AP-1 plays a key role in vascular SMC proliferation and phenotypic modulation. Our findings that Apo inhibits bFGF-induced increases in AP-1 promoter activity are consistent with previous reports of AP-1 involvement in oxidative stress [75,79], and provide evidence of a novel mechanism for NOX regulation of coronary smooth muscle KCNN4. It is important to note that while a previous study by Lapperre et al. (1999) showed upregulation of AP-1 activity following Apo treatment alone [80], our results are similar to a more recent study showing Apo inhibits pro-inflammatory factor-induced AP-1 and NF-B activation [81].

While it is clear that NOX plays a role in cardiovascular disease, including atherosclerosis, the role of the individual NOX isoforms is not as clear. Our results indicate that similar to the human cardiovascular system, porcine coronary smooth muscle expresses NOX1, NOX2, NOX4 and NOX5. Consistent with previous reports, in the basal state NOX4 expression was significantly greater than the other isoforms [43,82,83]. To our knowledge this is the first study to show the NOX isoform expression profile of porcine coronary smooth muscle and is of particular interest considering rodents do not express NOX5 [44,53].

Unlike the other NOX isoforms, NOX5 is composed of four cytosolic EF-hands with Ca$^{2+}$ binding sites resulting in Ca$^{2+}$ dependent regulation without the need of additional cytosolic subunits for activation [43,52,53,84]. Studies have shown that NOX5 activity and expression is regulated by NF-kB, AP-1, and STAT1/STAT3, and is increased by growth factors including PDGF [52,85,86]. NOX5 is thought to be an important regulator of numerous vascular pathologies playing a role in cell proliferation, cell growth, and signal transduction [43,87]. Combined with previous findings that NOX5 expression is elevated in atherosclerotic plaques [51,53], our novel findings that knockdown of NOX5 prevents bFGF upregulation of KCNN4 mRNA expression provide additional support that NOX5 plays a role in CVD by regulating vascular SMC proliferation and migration.

Although we demonstrated a complete inhibition of KCNN4 upregulation with NOX5 knockdown, we examined the potential role of other isoforms. NOX2 is the most widely distributed isoform and was shown to play a role in neointimal formation.

**Figure 9. Knockdown of NOX5 prevented coronary smooth muscle cell migration.** Transduction with AdNOX5shRNA significantly reduced chemotactic-induced migration in CSMCs compared to control adenovirus. Addition of TRAM-34 (100 nM), inhibited migration ~50% in control adenovirus cells, but had no effect in AdNOX5shRNA cells. Cell number per 20X field, n = 6–8. *P<0.05 vs other experimental groups.
doi:10.1371/journal.pone.0105337.g009
and restenosis [50,84,88], however siRNA knockdown of NOX2 did not prevent bFGF upregulation of KCNN4 mRNA expression in our studies. Consistent with previous reports that NOX4 is downregulated during vascular growth and is thought to be primarily responsible for basal cellular O$_2^-$ production [9,26,44,89], our findings demonstrate that knockdown of NOX4 does not prevent bFGF upregulation of KCNN4 mRNA expression in CSMCs. These results support previous findings that while increasing ROS production through an NADPH oxidase dependent pathway, bFGF does not upregulate the NOX2 or NOX4 isoforms in human or mouse endothelial cells [90]. Inhibition of NOX1, implicated in the pathogenesis of restenosis and atherosclerosis, has been shown to significantly inhibit neointimal formation [26,43,48,91], and unlike NOX2 and NOX4, has been shown to be upregulated by bFGF in rat and mouse aortic SMCs [92]. Unfortunately we were unable to successfully knockdown NOX1 in porcine CSMCs due to the lack of a complete mRNA sequence for porcine NOX1, thus preventing us from completely ruling out a contribution of NOX1 in bFGF upregulation of KCNN4 mRNA expression.

It is important to note, however, that none of the above mentioned studies examined the role of NOX5 in bFGF-induced ROS production. This is critical considering individual cells express multiple NOX isoforms suggesting separate distinct functions based on subcellular localization, cell type, and stimulus. The individualized functions of the NOX isoforms along with our data demonstrating a complete block of KCNN4 upregulation by NOX5 knockdown and high specificity of NOX5 mRNA knockdown by AdNOX5shRNA do, however, strongly suggest little to no role for other isoforms including NOX1 in bFGF-induced upregulation of KCNN4 in porcine CSMCs.

Based on previous studies looking at the mechanisms of mitogen-induced NOX activation, the potential signaling pathways involved in NOX5 dependent upregulation of KCNN4 in response to bFGF are likely complex and may involve positive feedback loops. bFGF is known to act through a number of signaling pathways including the ras/raf/MEK/ERK/MAPK cascade, the PI3K/IP3 cascade, and a pathway involving PI3K, PKC, and Rac [73,93,94]. Although known to result in increased NADPH oxidase activity, the PI3K pathway likely doesn’t play a considerable role in our particular study considering NOX5 activation is calcium sensitive and does not involve cytosolic subunits such as Rac [53].

The novel findings of our study, combined with results from previous studies showing KCNN4 plays a key role in regulating SMC phenotypic modulation and contributes to stenosis, we conducted chemotaxis assays to determine if NOX5 plays a role in migration. Consistent with previous studies indicating ROS play a role in atherosclerosis and restenosis [26,27], our findings demonstrate that knockdown of NOX5 inhibits mitogen-induced CSMC migration.

The PIP2 pathway is of interest though as it has been shown to result in increased cytosolic Ca$^{2+}$ [95], which is known to activate NOX5 [43,51] as well as KCNN4. Interestingly, studies have shown that IP3 sensitivity is increased by ROS [96,97] providing a mechanism by which increased NOX5 dependent ROS production may stimulate continued activation of NOX5 and continued release of Ca$^{2+}$ through a positive feedback loop. Another pathway of interest that may be working in conjunction with the PIP2/IP3 cascade is the ras-MAPK signaling pathway. Shown to be activated by growth factors and ROS, and thus potentially NOX5, the MAPK pathway involves activation of the c-jun and c-fos transcription factors inducing AP-1 activity known to be involved in KCNN4 upregulation [23,98,99]. KCNN4 activation results in hyperpolarization causing calcium influx [14], which may in turn activate NOX5 as well as further activating KCNN4 through a positive feedback loop.

Based on our previous studies showing upregulation of KCNN4 mediates coronary smooth muscle phenotypic modulation and contributes to stenosis, we conducted chemotaxis assays to determine if NOX5 plays a role in migration. Consistent with previous studies indicating ROS play a role in atherosclerosis and restenosis [26,27], our findings demonstrate that knockdown of NOX5 inhibits mitogen-induced CSMC migration.

The Acknowledgments

We gratefully acknowledge Rebecca Shaw, Miles Tanner, and Jennifer Casali for technical help.

The Author Contributions

Conceived and designed the experiments: HKAG DLT DKB. Performed the experiments: HKAG DLT DKB. Analyzed the data: HKAG DLT DKB. Contributed reagents/materials/analysis tools: HKAG DLT DKB. Wrote the paper: HKAG DLT.

References

1. Lopponow H, Werlan K, Buerke M (2006) Invited review: Vascular cells contribute to atherosclerosis by cytokine- and innate-immunity-related inflammatory mechanisms. Innate Immun 10: 63–87.
2. Aliev G, Castellani RJ, Petersen RB, Burnstlock G, Perry G, et al. (2004) Pathobiology of familial hypercholesterolemic atherosclerosis. J Submicrosc Cytol Pathol 36: 225–290.
3. San Martin A, Foncea R, Laurindo FR, Ebensperger R, Griendling KK, et al. (2007) Nox1-based NADPH oxidase-derived superoxide is required for VSMC activation by advanced glycation end-products. Free Radic Biol Med 42: 1671–1679.
4. Sung HJ, Eskin SG, Sakurai Y, Yee A, Kataoka N, et al. (2005) Oxidative stress produced with cell migration increases synthetic phenotype of vascular smooth muscle cells. Ann Biomed Eng 33: 1546–1554.
5. Bef inflammation are important in the regulation of contractile smooth muscle cell phenotype: implications for vascular smooth muscle cells. Am J Physiol Cell Physiol 286: C609–C626.
6. Clempus RE, Sorescu D, Dikalova AE, Pounkova L, Jo P, et al. (2007) Nox1 is required for maintenance of the differentiated vascular smooth muscle cell phenotype. Arterioscler Thromb Vasc Biol 27: 42–49.
7. Kawai-Kowase K, Owens GK (2007) Multiple repressor pathways contribute to phenotypic switching of vascular smooth muscle cells. Am J Physiol Cell Physiol 292: 816–895.
8. Owens GK, Kumar MS, Wamhoff BR (2004) Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev 84: 767–801.
9. Yoshida T, Gan Q, Shang Y, Owens GK (2007) Platelet-derived growth factor-BB regulates smooth muscle cell marker genes via changes in binding of MLK factors and histone deacetylases to their promoters. Am J Physiol Cell Physiol 292: 816–895.
10. Beamish JA, He P, Konte-Marchant K, Marchant RE. (2010) Molecular regulation of contractile smooth muscle cell phenotype: implications for vascular tissue engineering. Tissue Eng Part B Rev 16: 467–491.
11. Tharp DL, Bowles DK (2009) The intermediate-conductance Ca$^{2+}$-activated K$^+$ channel (KcA3.1) mediates phenotypic modulation of coronary smooth muscle. Am J Physiol Heart Circ Physiol 291: 2493–2503.
15. Kehler R, Wulff H, Echter J, Kneifel M, Neumann D, et al. (2003) Blockade of the intermediate-conductance calcium-activated potassium channel as a new therapeutic strategy for restenosis. Circulation 108: 1119–1125.

16. Si H, Gregic I, Heyken WT, Maier T, Hoyer J, et al. (2006) Mitogenic modulation of Ca^2+-activated K^+ channels in proliferating A7r5 vascular smooth muscle cells. Br J Pharmacol 148: 909–917.

17. Toyama K, Wulff H, Chandy KG, Azam P, Raman G, et al. (2008) The intermediate-conductance calcium-activated potassium channel KCa3.1 contributes to angiogenesis in mice and human cells. J Clin Invest 118: 3025–3037.

18. Tharp DL, Wamhoff BR, Wulff H, Raman G, Cheong A, et al. (2008) Local delivery of the KCa3.1 blocker, TRAM-34, prevents acute angioiastibulated coronary smooth muscle phenotypic modulation and limits stenosis. Arterioscler Thromb Vasc Biol 28: 1084–1089.

19. Ghanshani S, Wulff H, Miller MJ, Rohm H, Nebeh A, et al. (2000) Upregulation of the KCa1 potassium channel during T-cell activation. Molecular and functional consequences. J Biol Chem 275: 37317–37319.

20. Cheong A, Bingham AJ, Li J, Kumar B, Sukumar F, et al. (2005) Downregulation of the TRPM7 ion channel is a switch enabling critical potassium channel expression and cell proliferation. Mol Cell 20: 45–52.

21. Cheong A, Wood IC, Beech DJ (2006) Less REST, more vascular disease? Regulation of cell cycle and migration of vascular smooth muscle cells. Cell Cycle 5: 129–135.

22. Zhan Y, Kim S, Yasumoto H, Namba M, Miyazaki H, et al. (2002) Effects of dominant-negative c-Jun on platelet-derived growth factor-induced vascular smooth muscle cell proliferation. Arterioscler Thromb Vasc Biol 22: 62–68.

23. Adams M, Nishida C, Lehmann U, Hall JL (2006) Transcription factor and kinase-mediated signaling in atherosclerosis and vascular injury.Curr Atheroscler Rep 8: 252–260.

24. Angel P, Karin M (1991) The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. Biochem Biophys Acta 1192: 129–157.

25. Curran T, Franza BR Jr (1988) Fos and Jun: the AP-1 connection. Cell 55: 395–398.

26. Cav AE, Brewer AC, Narayananapichakker A, Ray A, Grieve DJ, et al. (2006) NADPH oxidases in cardiovascular health and disease. Antioxid Redox Signal 8: 691–728.

27. Greendl KK, Fitz Gerald GA (2003) Oxidative stress and cardiovascular injury part II: animal and human studies. Circulation 108: 2034–2040.

28. Fike CD, Slaughter JC, Kaplowitz MR, Zhang Y, Aschner JL (2008) Reactive oxygen species: mechanisms of activation. Cardiovasc Res 65: 16–27.

29. Lin FY, Chen YH, Tsai JS, Chen JW, Yang TL, et al. (2006) Endotoxin induces toll-like receptor 4 expression in vascular smooth muscle cells via NADPH oxidase activation and mitogen-activated protein kinase signaling pathways. Arterioscler Thromb Vasc Biol 26: 2603–2607.

30. Lye AL, Greendl KK (2006) Modulation of vascular smooth muscle signaling by reactive oxygen species. Physiol Rev 21: 269–280.

31. Brandes RP, Kreuzer J (2005) Vascular NADPH oxidases: molecular mechanisms of activation. Cardiovasc Res 65: 16–27.

32. Jiang F, Zhang Y, Dusting GJ (2011) NADPH oxidase-mediated redox signaling: role in cellular stress response, stress tolerance, and tissue repair. Pharmacol Rev 63: 61–122.

33. Trachootham D, Lu W, Ogasawara MA, Nila RD, Huang P (2008) Redox regulation of cell survival. Annu Rev Pharmacol Toxicol 48: 1343–1374.

34. Cain H, Greendl KK, Harrison DG (2003) The vascular NADPH oxidases as therapeutic targets in cardiovascular diseases. Trends in Pharmacol Sci 24: 471–478.

35. Brown DJ, Greendl KK (2009) N ox proteins in signal transduction. Free Radic Biol Med 47: 1239–1253.

36. Lassegue B, Climpus RE (2003) Vascular NADPH oxidases: specific features, expression, and regulation. Am J Physiol Integ Comp Physiol 285: 277–297.

37. Diebold I, Perry A, Hess J, Gorlach A (2010) The NADPH oxidase subunit NOX1 is a new target gene of the hypoxia-inducible factor-1. Mol Biol Cell 21: 2087–2096.

38. Szoc K, Lassegue B, Sorensen D, Hilenski LL, Valgus L, et al. (2002) Upregulation of Nox-based NADPH oxidases in restenosis after carotid injury. Am J Physiol Lung Cell Mol Physiol 282: 21–27.

39. Dikalova AE, Gongora MC, Harrison DG, Lambeth JD, Dikalov S, et al. (2010) Upregulation of Nox1 in vascular smooth muscle leads to impaired endothelium-dependent relaxation via eNOS uncoupling. Am J Physiol Heart Circ Physiol 298: H743–H753.

40. Fan C, Katsumaya N, Nishinaka T, Yah-Nishimura C (2005) Transactivation of the EGF receptor and a PI3 kinase-AKT-1 pathway is involved in the upregulation of NOX1, a catalytic subunit of NADPH oxidase. FEBS Letters 570: 1301–1305.

41. Sorensen D, Weiss B, Lassegue B, Climpus RE, Szoc K, et al. (2002) Superoxide production and expression of Nox family proteins in human atherosclerosis. Circulation 105: 1429–1435.

42. Li J, Newburger PE, Goumis MJ, Dargan P, Zhang X, et al. (2010) Local arterial nanoparticle delivery of siRNA for NOX2 knockdown to prevent restenosis in an atherosclerotic rat model. Gene Ther 17: 1279–1287.

43. Guzik TJ, Chen W, Gongora MC, Guzik B, Lob HE, et al. (2008) Calcium-dependent adenosine monophosphate (cAMP) phosphodiesterase type 1 contributes to vascular oxidative stress in human coronary artery disease. J Am Coll Cardiol 52: 1803–1809.

44. Jay DR, Papaharalambus CA, Seidel-Rogol B, Dikalova AE, Lassegue B, et al. (2008) Nox5 mediates PDGF-induced proliferation in human aortic smooth muscle cells. Free Radic Biol Med 45: 329–335.

45. Schulz E, Munzel T (2008) NOX5, a new ‘‘radical’’ player in human atherosclerosis? J Am Coll Cardiol 52: 1010–1012.

46. Wamhoff BR, Bowles DK, Ezhilarasan D, Sinha S, Sompyo AP, et al. (2004) Type- voltage-gated Ca2+ channels modulate expression of smooth muscle differentiation marker genes via a rho kinase/myocardin/SRF-dependent mechanism. Circ Res 95: 406–414.

47. Dikalova S, Greendl KK, Harrison DG (2007) Measurement of reactive oxygen species in cardiovascular studies. Hypertension 49: 717–727.

48. Munzel T, Afanas’ev IB, Kleshevich AL, Harrison DG (2002) Detection of superoxide in vascular tissue. Arterioscler Thromb Vasc Biol 22: 1741–1748.

49. Greendl H, Wang G, Gimpl G, Amrhein J, Zhou P, et al. (2009) NMDA receptor activation increases free radical production through nitric oxide and NOX2. J Neurosci 29: 2545–2552.

50. Bowles DK, Madelblat KK, Ganjam VK, Rubin LJ, Tharp DL, et al. (2004) Endogenous testosterone increases L-type Ca2+ channel expression in porcine coronary smooth muscle. Am J Physiol Heart Circ Physiol 287: 2091–2098.

51. Bowles DK, Heaps CL, Turk JR, Maddalib KK, Price EM (2004) Hypercholesterolemia inhibits L-type calcium current in coronary macro-, not microcirculation. J Appl Physiol 96: 2240–2244.

52. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2 (delta)delta CT method. Methods 25: 402–408.

53. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data using the comparative CT method. Nat Protoc 3: 1101–1108.

54. Bowles DK, Laughlin MH, Sturck M (1998) Exercise training increases K+ channel contribution to regulation of coronary arterial tone. J Appl Physiol 84: 1225–1233.

55. Heaps CL, Tharp DL, Bowles DK (2005) Hypercholesterolemia abolishes voltage-dependent K+ channel contribution to adenosine-mediated relaxation in coronary arterioles. Am J Physiol Heart Circ Physiol 288: 568–578.

56. Wulff H, Miller MJ, Hanwel M, Grunser S, Caballan MD, et al. (2000) Design of a potent and selective inhibitor of the intermediate-conductance Ca2+-activated K+ channel, IKCa1: a potential immunosuppressor. Proc Natl Acad Sci USA 97: 8151–8156.

57. Terashima M, Ohashi Y, Awano K, Azumi H, Otsui K, et al. (2009) Impact of angiotensin II on coronary plaque formation and NAD(P)H oxidase-derived reactive oxygen species in patients with coronary artery disease. Circulation 119: 2087–2096.

58. Taniyama Y, Griendling KK (2003) Reactive oxygen species in the vasculature: mechanisms of activation. Cardiovasc Res 71: 216–225.
72. Tony M (2008) Apocynin, NADPH oxidase, and vascular cells: a complex matter. Hypertension 5: 172–174.
73. Gregic I, Eichler I, Heinau P, Si H, Brakemeier S, et al. (2003) Selective blockade of the intermediate-conductance Ca2+-activated K+ channel suppresses proliferation of microvascular and macrovascular endothelial cells and angiogenesis in vivo. Arterioscler Thromb Vasc Biol 23: 704–709.
74. Trebak M, Gunnam R, Singer HA, Jouffredheil D (2010) Interplay between calcium and reactive oxygen/nitrogen species: an essential paradigm for vascular smooth muscle signaling. Antioxid Redox Signal 12: 637–674.
75. Rao GN, Katki KA, Madamanchi NR, Wu Y, Birrer MJ (1999) JunB forms the majority of the AP-1 complex and is a target for redox regulation by receptor tyrosine kinase and G protein-coupled receptor agonists in smooth muscle cells. J Biol Chem 274: 6003–6010.
76. Hansen DG, Cai H, Landmerss U, Griendling KK (2002) Interactions of angiotensin II with NADPH oxidase, oxidant stress and cardiovascular disease. J Renin Angiotensin Aldosterone Syst 4: 51–61.
77. Giordano FJ (2005) Oxygen, oxidative stress, hypoxia, and heart failure. J Clin Invest 115: 500–508.
78. Griendling KK, Sorescu D, Lassegue B, Ushio-Fukai M (2000) Modulation of NOx pathways activated by angiotensin II with NADPH oxidase, oxidant stress and cardiovascular disease. Antioxid Redox Signal 105: 209–210.
79. Yamakawa T, Tanaka S, Yamakawa Y, Kamei J, Numaguchi K, et al. (2002) Lysophosphatidylcholine activates extracellular signal-regulated kinases 1/2 through reactive oxygen species in rat vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 22: 752–758.
80. Lappert TS, Jimenez LA, Antonicelli F, Drost EM, Hiemstra PS, et al. (1999) Apocynin increases glutathione synthesis and activates AP-1 in alveolar epithelial cells. FEBS Lett 443: 235–239.
81. Kim SY, Moon KA, Jo HY, Seon SH, et al. (2012) Anti-inflammatory effects of apocynin, an inhibitor of NADPH oxidase, in airway inflammation. Immuno Cell Biol 49(4): 441–448.
82. Dikalov SI, Dikalova AE, Bikineyeva AT, Schmidt HHHW, Harrison DG, et al. (2008) Distinct roles of Nox1 and Nox4 in basal and angiotensin II-stimulated superoxide and hydrogen peroxide production. Free Radic Biol Med 45: 1340–1351.
83. Miller FJ Jr (2009) NADPH oxidase 4: walking the walk with Poldip2. Circ Res 105: 209–210.
84. Trebak M, Ginnan R, Singer HA, Jouffredheil D (2010) Interplay between calcium and reactive oxygen/nitrogen species: an essential paradigm for vascular smooth muscle signaling. Antioxid Redox Signal 12: 637–674.
85. Montezano A, Burger D, Paravicini T, Chignalia A, Yusuf H, et al. (2010) JunB forms the majority of the AP-1 complex and is a target for redox regulation by receptor tyrosine kinase and G protein-coupled receptor agonists in smooth muscle cells. J Biol Chem 274: 6003–6010.
86. Ushio-Fukai M, Alexander RW, Akes M, Griendling KK (1998) p38 Mitogen-activated protein kinase is a critical component of the redox-sensitive signaling pathways activated by angiotensin II. Role in vascular smooth muscle cell hypertrophy. J Biol Chem 273: 15022–15029.