Calcium/Calmodulin-dependent Kinase II mediates the Phosphorylation and Activation of NADPH Oxidase 5


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Running head: Activation of Nox5 by CAMKII

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Number of text pages: 30
Number of tables: 1
Number of figures: 6
Number of References: 40
Number of Words - Abstract: 249
Number of Words - Introduction: 706
Number of Words - Discussion: 1306

List of Abbreviations: CAMKII, Calcium and calmodulin activated kinase II; Nox, NADPH oxidase; eNOS, endothelial nitric oxide synthase;; ROS, reactive oxygen species;; RLU, relative light units;
Abstract

Excessive synthesis of reactive oxygen species (ROS) contributes to the pathology of many human diseases and originates from changes in the expression and posttranslational regulation of the transmembrane NADPH oxidases (Nox). Nox5 is a novel Nox isoform whose activity is regulated by intracellular calcium levels. We recently reported that the activity and calcium-sensitivity of Nox5 can also be modulated by direct phosphorylation. However, the kinases that phosphorylate Nox5 have not been identified and thus the goal of this study was to determine if calcium activated kinases such as CAMKII are involved. We found that Nox5 activity in BAEC was suppressed by 2 doses of the CAMKII inhibitor, KN-93. In co-transfected COS-7 cells, WT and constitutively active CAMKII, but not a dominant negative, robustly increased basal Nox5 activity. The ability of CAMKII to increase Nox5 activity was also observed with fixed calcium concentrations in an isolated enzyme activity assay. CAMKII did not elevate intracellular calcium or activate other Nox enzymes. In vitro phosphorylation assays revealed that CAMKII can directly phosphorylate Nox5 on T494 and S498 as detected by phosphorylation state-specific antibodies. MS analysis revealed the phosphorylation of additional, novel sites at S475, S502 and S675. Of these phosphorylation sites, mutation of only S475 to alanine prevented CAMKII-induced increases in Nox5 activity. The ability of CAMKIIα to phosphorylate S475 in intact cells was supported by the binding of Nox5 to phosphoprotein-affinity columns and via MS/MS analysis. Collectively these results suggest that CAMKII can positively regulate Nox5 activity via the phosphorylation of S475.
MOL #70193

Introduction

NADPH oxidases (Noxes) are major sources of reactive oxygen species (ROS) including superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) in mammalian cells (Beckman and Ames, 1998; Lambeth, 2004). Because of their inherent reactivity, ROS production is tightly regulated to control the appropriate amount at the right time and place. In response to physiological stimuli, ROS are important contributors to cellular processes such as host defense, cell signaling, smooth muscle contraction, differentiation and the formation of otoconia (Lambeth, 2007; Nakano et al., 2008). In contrast, the excessive or inappropriate production of ROS has been shown to underlie many disease processes such as asthma, cancer, atherosclerosis, hypertension and diabetes (Lambeth, 2007). Strategies to suppress ROS using antioxidants have proven largely ineffective in the treatment of cancer and heart disease. The selective blockade of pathological or excessive ROS production, without targeting all ROS, may be a more effective approach. To achieve this, a better understanding of the mechanisms controlling Nox activity is necessary.

Nox enzymes can be functionally divided into an N-terminal transmembrane domain that spans the membrane 6 times and supports two heme residues, and a C-terminal reductase domain that binds FAD and NADPH. The Nox family of enzymes is comprised of 7 members, designated Nox1-5 and Duox1-2 that are found in both phagocytic and non-phagocytic cells (Bedard and Krause, 2007). The mechanisms governing the activity of these isoforms is incompletely understood and an area of intensive investigation (Bedard and Krause, 2007). Activation of Nox1-3 requires the translocation of a number of
cytosolic subunits. In contrast, Nox4 and Nox5 do not require cytosolic subunits to synthesize ROS, and Nox4 has been shown to constitutively produce \( H_2O_2 \) instead of \( O_2^- \) (Martyn et al., 2006).

Nox5 was the last Nox isoform to be identified. Although originally described in testes and lymph nodes, it is also expressed in vascular tissue where it promotes the proliferation of vascular smooth muscle cells (Jay et al., 2008; Montezano et al., 2010). The expression and activity of Nox5 are dramatically elevated in coronary arteries with advanced lesions which suggests that Nox5 may contribute to vascular disease (Guzik et al., 2008). Nox5 contains a unique N-terminus that is characterized by 4 EF-hands that bind calcium and regulate its activity (Fulton, 2009). In addition, the activity of Nox5 can be also regulated by direct phosphorylation in response to PMA (Jagnandan et al., 2007; Serrander et al., 2007). PKC is thought to mediate the phosphorylation of Nox5 on T494 and S498, but this is based on the use of pharmacological inhibitors and the isoforms involved remain to be indentified (Jagnandan et al., 2005). Calmodulin has also been shown to activate Nox5 at lower levels of intracellular calcium (Tirone and Cox, 2007). However, the role of other kinase(s) in the phosphorylation and activation of Nox5, particularly those sensitive to calcium-calmodulin, are poorly understood.

Elevation of intracellular calcium influences a multitude of cellular functions (Berridge et al., 2003). Some of these effects are mediated by multifunctional calcium/calmodulin dependent protein kinases (CAMKs), a family of serine/threonine kinases that includes CAMKII (Soderling, 1999). CAMKII is
activated by cytosolic calcium which promotes calmodulin binding and autophosphorylation of T286 (Griffith, 2004). A number of studies have identified close relationships between CAMKII signaling, ROS production and vascular function. Elevated ROS can render CAMKII constitutively active via oxidation of methionines 281/282 (Erickson et al., 2008). In human macrophages, zymosan induced ROS production, predominantly generated by Nox2, is dependent on CAMKII signaling pathways (Kelly et al., 2010). CAMKII has also been shown to mediate redox-sensitive gene regulation in vascular endothelium (Cai et al., 2001). Interestingly, H₂O₂ has been shown to positively regulate Nox5 activity via a Ca²⁺ mediated, redox dependent signaling pathway (El Jamali et al., 2008).

The ability of calcium-dependent kinases to regulate Nox5 activity is not yet known. A previous study reported that CAMKII inhibitors do not alter Nox5 activity in response to PMA (Serrander et al., 2007). However, the inhibitor employed was an inactive structural analog (KN-92) and PMA does not elevate intracellular calcium. Therefore, whether an active CAMKII inhibitor (KN-93) can influence Nox5 activity or whether CAMKII is important following calcium-mobilization remain unanswered questions. Therefore, the goals of the current study are to investigate whether CAMKII can influence the phosphorylation and activity of Nox5.
Material and Methods

Cell culture and Transfection

COS-7 cells and bovine aortic endothelial cells (BAEC) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing L-glutamine, penicillin, streptomycin, and 10% (v/v) fetal bovine serum. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen). KN-93 (N-[2-[[3-(4-chlorophenyl)-2-propen-1-yl]methylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4-methoxy-benzenesulfonamide, CAS 139298-40-1) was obtained from Cayman Chemical. Control and human CAMKIIα – specific siRNA were experimentally verified sequences that were obtained from Qiagen (Krueger et al., 2007) and checked for homology to all other sequences of the genome using a nonredundant database and designed with HP OnGuard which provides asymmetry (Schwarz et al., 2003), 3’ UTR/seed region analysis (Grimson et al., 2007) SNP avoidance and interferon motif avoidance (Judge et al., 2005) to maximize target specificity. Human aortic vascular smooth muscle cells were obtained and cultured in SMbM media from Lonza.

Immunoprecipitation and Immunoblotting

COS-7 cells were lysed in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM NaF, 15 mM Na4P2O7, 1 mM Na3VO4, 1% v/v Triton X-100, 1 mM phenylmethlsulfonyl fluoride, 10 µg/ml pepstatin A, and 5 µg/ml aprotinin. Lysates were be centrifuged at 10,000 x g to concentrate insoluble material. Nox5 was extracted from detergent resistant microdomains by the addition of 1% SDS and
subsequently diluted 1:10 in lysis buffer. Protein extracts were precleared by incubation with Protein A/G-agarose for 2 h at 4º C with rocking. Agarose beads were then be pelleted by centrifugation at 1,000 x g. HA-Nox5 in precleared lysates was immunoprecipitated by incubation with preconjugated agarose:anti-HA antibody overnight at 4º C with rocking. Immunoprecipitated proteins were eluted from the beads by boiling for 5 min in 2X sample buffer. Immunoprecipitates or cell lysates were immunoblotted with various antibodies.

**Purification of Phosphorylated Proteins from Intact Cells**

COS-7 cells were co-transfected with WT or S475A Nox5 in the presence of CAMKIIα and 48 hours later, cells were lysed in RIPA buffer. Lysates were diluted in binding buffer in the absence of metal chelators and reducing agents, and phosphoproteins were purified using phosphoaffinity columns (Thermo Scientific).

**In vitro Phosphorylation**

Nox5 was isolated by immunoprecipitation from COS-7 cells transduced with HA-Nox5 adenovirus and incubated with 50ng of active CAMKII (Cell Signalling Technology) for 20 min at 30º C in buffer containing 20 mM HEPES, pH 7.4, 10 mM MgCl₂, 10mM MnCl₂, 0.5mM CaCl₂, 1µg CAM, and 1mM DTT with or without 100 µM ATP. The reaction was terminated by the addition of SDS sample buffer. Incorporation of phosphate into Nox5 was determined using by SDS-PAGE followed by immunoblotting using
phosphorylation state specific antibodies that recognize phosphorylated Nox5 at S490, T494 and S498 as previously characterized (Jagnandan et al., 2007).

**MS analysis**

*In-vitro phosphorylated Nox5* - Immunoprecipitated Nox5 was phosphorylated in vitro as described above, size fractionated by SDS-PAGE and proteins visualized by silver staining. The band of interest was excised from the gel and digested with trypsin (0.1 µg) for 45 min at 4°C. Peptides were then extracted from the gel at room temperature and analyzed by MS using a LTQ Orbitrap (Thermo Scientific). Protein identification was obtained from the MS/MS spectra using Mascot analysis software (Matrix Science, Boston, MA).

*Phosphorylated Nox5 in intact cells* - HA-NOX5β was purified using immunoprecipitation from COS-7 cells that were transfected with NOX5β and CAMKIIα. Immune complexes were subjected to SDS-PAGE (7% acrylamide). Proteins were visualized using the Imperial protein stain (Thermofisher) and a band corresponding to the molecular weight of NOX5β (~82kDa) was excised, destained and subjected to overnight in-gel digestion with trypsin (25 ng/µl in 25mM ammonium bicarbonate buffer, pH 7.8). Peptides were extracted with 0.1% TFA/75% acetonitrile and evaporated to near dryness. Peptide calibration standards and matrix CHCA were purchased from Applied Biosystems. All spectra were taken on an ABSciex 5800 MALDI-TOF Mass Spectrometer in positive reflector mode (10kV) with a matrix of CHCA. At least 1000 laser shots were averaged to get each spectrum. Masses were calibrated to known peptide
standards. 5µl aliquots of the extracted tryptic digest were purified using C18 ZipTip columns (Millipore) as per manufacturer’s instructions. Bound peptides were desalted with two 5µL washes of 0.1% TFA and then eluted with 2.5 µL of aqueous, acidic acetonitrile (75% CH3CN, 0.1% TFA). The eluate was mixed with 2.5µL freshly prepared CHCA stock solution (20 mg/ml CHCA in aqueous acetonitrile), and 1.5 µL aliquots were spotted onto a MALDI sample plate for air drying. MS/MS of 1822.74m/z peak was done in positive reflector mode without CID. MS and MS/MS spectra were analyzed using the Mascot Distiller software package.

Measurement of Reactive Oxygen Species

COS-7 cells were transfected with cDNAs encoding Nox5 or control plasmids (RFP or lacZ) and 24 hours later cells were re-plated into white TC-treated 96-well plates (ThermoLabsystems) at a density of approximately 5x10^4 cells per well. The cells were incubated at 37ºC in phenol free DMEM (Sigma) containing 400µM of the luminol analogue L-012 (Wako) for a minimum of 20 minutes prior to the addition of agonists (Jagnandan et al., 2007). Luminescence was quantified over time using a Lumistar Galaxy (BMG) luminometer. The specificity of L-012 for reactive oxygen species was confirmed by transfecting cells with a control plasmid such as GFP or lacZ or by co-incubation of a superoxide scavenger such as Tiron (5mM). Both of these interventions yielded virtually undetectable levels of luminescence under control, PMA or ionomycin stimulated conditions. Superoxide production is recorded as relative light units (RLU) and as
such the absolute levels of ROS in separate experiments are not directly comparable. In human aortic smooth muscle cells, the calcium-dependent release of superoxide was measured as described by others using media with reduced calcium levels (Montezano et al., 2010).

Isolated Nox5 Activity Assay

COS-7 cells co-expressing Nox5 and CAMKIIα were lysed in MOPS (30mM, pH 7.2) based buffer containing KCl (100mM), Triton (0.3%) and protease inhibitors (Sigma). Adherent cells were rocked gently, the lysis buffer aspirated, and then the cells were washed 3 times with PBS (4°C). Remaining cytoskeletal fractions were resuspended in the above MOPS buffer, sonicated at low power and spun down at 14,000 rpm (4°C). The supernatant was then aspirated and the pellet was resuspended in MOPS buffer with mild sonication. The cell free extract was aliquoted into buffers containing, L012 (400µM), 1mM MgCl₂, 100µM FAD (Sigma) and 26µM of Calcium chloride. After a brief period of equilibration, reduced NADPH (Sigma) was injected to a final concentration of 100µM and the production of reactive oxygen species was monitored over time.

Calcium Measurements

Change in calcium concentrations were measured under resting conditions and following exposure to ionomycin using Fluo4-NW (Invitrogen) as described previously (Church and Fulton, 2006).
Statistics: All statistical analyses were performed using Instat software and were made using a two tailed student’s t-test or ANOVA with a post-hoc test where appropriate. Differences are considered significant at p <0.05.
Results

Endogenous CAMKII positively regulates Nox5 activity

To determine whether CAMKII has a role in the regulation of Nox5 activity, we first utilized a pharmacological inhibitor of CAMKII, KN-93. Bovine aortic endothelial cells (BAEC) were used as a source of endogenous CAMKII (Fleming et al., 2001) and were transduced with a Nox5 adenovirus as these cells express low amounts of Nox5 compared to native blood vessels (unpublished observation). As shown in Figure 1A, pretreatment of BAEC with different doses of the CAMKII inhibitor KN-93, progressively reduced superoxide production from Nox5. We next investigated a role for CAMKII in the regulation of ROS production in human aortic vascular smooth muscle cells (HASMC), which are known to endogenously express Nox5 (Jay et al., 2008). As shown in Figure 1B, silencing CAMKIIα expression reduced calcium-dependent ROS production in HAVSMC.

Active CAMKIIα stimulates Nox5 activity

To complement results obtained using pharmacological inhibitors and siRNA, we next utilized a genetic approach to determine whether CAMKII is sufficient to increase Nox5 activity. COS-7 cells were co-transfected with Nox5 and either a control gene (RFP) or WT-CAMKIIα and superoxide release was measured. As shown in Figure 2A, cells expressing WT-CAMKIIα released significantly more superoxide versus control cells. The equal expression level of Nox5 (lower panel) in the presence of CAMKIIα suggests that the increase in activity results from a
post-translational modification. The ability of CAMKII to increase Nox5-derived superoxide was sensitive to pharmacological inhibition with KN-93 (Figure 2B). To further explore a relationship between Nox5 and CAMKII, we co-transfected COS-7 cells with Nox5 and either a control cDNA (RFP), WT, constitutively active or a dominant negative CAMKIIα. As shown in Figure 2C, co-expression of WT-CAMKII increases Nox5 activity and superoxide release. Co-expression of a constitutively active form of CAMKII (T286D), which mimics the persistent phosphorylation of T286, with Nox5 produces significantly higher levels of superoxide than the WT. Co-expression of a dominant negative CAMKII (T305D), which mimics persistent inhibitory phosphorylation, does not elevate superoxide production above control levels.

**CAMKIIα directly modifies Nox5 activity**

We next assessed whether CAMKII-can directly influence Nox5 activity or alter other secondary events such as the level of intracellular calcium. To achieve this we first performed an isolated Nox5 activity assay. Nox5 was purified from COS-7 cells co-expressing a control cDNA (RFP) or CAMKIIα and reconstituted with calcium, FAD and superoxide production initiated with NADPH. As shown in Figure 3A, Nox5 enzymatic activity from cells co-expressing CAMKII produced more superoxide than Nox5 extracted from control cells in response to NADPH. This occurred despite an apparently lower level of Nox5 expression in cell-free extracts from cells expressing CAMKIIα (inset). To determine whether CAMKII influences the level of intracellular calcium, we incubated COS-7 cells co-
expressing Nox5 and either a control cDNA or CAMKIIα and measured intracellular calcium using the fluorescent calcium-reporter, Fluo-4. We found that expression of CAMKII did not modify basal or ionomycin-stimulated intracellular calcium levels (Figure 3B).

**CAMKII-does not stimulate ROS production from other Nox enzymes**

To address whether CAMKII can increase the activity of other Nox enzymes we co-expressed CAMKIIα with Nox1 and its regulatory proteins, NOXO1 and NOXA1. While CAMKII can potently increase superoxide release from Nox5, it does not modify Nox1 activity (Figure 3C).

**Nox5 is a substrate for CAMKII phosphorylation**

To determine whether CAMKII can directly phosphorylate Nox5, we performed an in vitro kinase assay using immunoprecipitated Nox5 as a substrate. As shown in Figure 4A, in the presence of ATP, recombinant CAMKIIα robustly phosphorylated Nox5 on Serine 498 (S498), threonine 494 (T494) but not on Serine 490. To identify whether these sites are functionally important we expressed single (S498A, T494A) or triple (S490A, T494A, S498A) phosphonull mutants of Nox5, together with CAMKIIα. As shown in Figure 4B-D, single mutation of Nox5 on T494 or S498 or mutation of all three phosphorylation sites did not prevent the ability of CAMKII to increase Nox5 activity.

**Identification and significance of novel Nox5 phosphorylation sites**
The ability of CAMKII to modify Nox5 activity appears to be due to a direct effect on the enzyme. However, the mutation of S490, T494 and S498 to non-phosphorylatable analogs did not prevent CAMKII-dependent changes in Nox5 activity. Therefore, we next examined whether CAMKII can phosphorylate Nox5 on other sites. Nox5 was phosphorylated in vitro using recombinant CAMKII, purified via SDS-PAGE, trypsin digested, and subjected to MS/MS analysis (Duval et al., 2007). We found an additional 3 sites of phosphorylation, serines 475, 659 and 502 listed by rank of Mascot and Sequest scores (Table 1). To address the functional significance of these sites, we made point mutations of serine 475, 659 and 502 to the non-phosphorylatable analogue alanine. As shown in Figure 5A, the Nox5 mutant S475A was active, but did not produce additional superoxide in the presence of CAMKII. The other identified sites, S659 and S502 were significantly activated by CAMKII, albeit to a reduced degree compared to the WT Nox5 (Figure 5A). We also directly compared the calcium-dependent activity of S475A, S502A, S659A mutants versus that of WT-Nox5 and found that the S475A Nox5 mutant produced less superoxide following stimulation with ionomycin (Figure 5B).

**Identification of Nox5 S475 phosphorylation in intact cells.**

Having shown that S475 is a site for CAMKIIα-mediated phosphorylation of Nox5 in vitro and that S475 is functionally relevant in mediating CAMKIIα-stimulated ROS production, we next sought evidence that this site is phosphorylated in intact cells. COS-7 cells expressing WT or S475A Nox5 together with CAMKIIα
were lysed and phosphorylated proteins in the lysates were bound to phosphoprotein affinity columns. The relative expression of WT and S475A Nox5 in total cell lysates is shown in Figure 6A. As shown in Figure 6B, a Western blot on the eluate from the phosphoprotein affinity columns revealed greater binding of WT-Nox5 as compared to the S475A phosphonull mutant. These results suggest that in cells, the CAMKIIα-induced phosphorylation of Nox5 is diminished by mutation of S475. To obtain direct evidence that S475 is phosphorylated in intact cells, we next performed MS analysis. Nox5 was immunoprecipitated from cells expressing WT-Nox5 and CAMKIIα, and size fractionated by SDS-PAGE. Bands corresponding to the MW of Nox5 were excised, trypsin digested and subjected to MS/MS analysis. As shown in Figure 6C, using MADI-TOF and MS/MS analysis we detected a phosphorylated peptide that corresponds to the region flanking S475.
Discussion

The major findings of this study are that CAMKII can directly increase the activity of Nox5 and promote superoxide release. The effect of CAMKII on superoxide release is selective for Nox5 as it does not modify the activity of Nox1. We found that in vitro CAMKIIα can phosphorylate Nox5 on at least 5 different sites, T494, S498, S502, S475 and S659. Of these sites, only S475 is functionally important for superoxide release. The ability of CAMKII to phosphorylate Nox5 on S475 in intact cells is suggested from the loss of binding to phosphoprotein affinity columns and is directly supported by the detection of a phosphorylated peptide containing S475 using mass spectrometry. Collectively these results suggest that CAMKIIα can promote superoxide release from cells, at least in part, by regulating the activity of Nox5.

Previously, we showed that the PMA-dependent phosphorylation of Nox5 on T494 and S498 increases the calcium-sensitivity of Nox5 and permits elevated superoxide release at resting levels of intracellular calcium (Jagnandan et al., 2007). While we found that CAMKII can increase the phosphorylation of Nox5 on T494 and S498 in vitro, the mutation of these sites to non-phosphorylatable residues had no effect on the ability of CAMKII to increase superoxide release from cells expressing Nox5. Thus within the cell, it is possible that either CAMKII does not modify the phosphorylation of these sites or alternatively that the phosphorylation of S475 can override these effects.

The mechanism by which the phosphorylation of S475 increases Nox5 activity is not yet known and the exact mechanisms regulating the calcium-
dependent activity of Nox5 in general are not fully understood (Wei et al., 2010). This site lies in the C-terminal, cytoplasmic region of Nox5, just after the predicted FAD binding region and before the first NADPH binding site (Fulton, 2009). This region also contains, in close proximity, the other Nox5 phosphorylation sites T494, S498 and S502. Thus, given its location it is likely that phosphorylation of this site promotes electron flow from NADPH to FAD or FAD to the heme moieties.

Within the cell, Nox5 can be found primarily on membranes of the endoplasmic reticulum and also at the plasma membrane (Jagnandan et al., 2007; Kawahara and Lambeth, 2008; Serrander et al., 2007). The mechanism by which CAMKII interacts with Nox5 is not yet known. CAMKII is not intrinsically membrane bound but can interact with substrates at the plasma membrane and also those that are present on the sarco/endoplasmic reticulum such as the ryanodine receptor (Hudmon et al., 2005; Wehrens et al., 2004). Some substrates of CAMKII have been proposed to contain a binding motif that can resemble part of the regulatory domain of CAMKII (Couchonnal and Anderson, 2008), but it is not yet known whether Nox5 binds tightly to CAMKII or participates in “kiss and run” interactions prior to phosphorylation.

CAMKII has been shown to regulate the levels of intracellular calcium by phosphorylating subunits of voltage gated calcium channels and also the ryanodine receptor (Hudmon et al., 2005; Wehrens et al., 2004). However, this action is unlikely to account for the ability of CAMKII to increase Nox5 activity in COS-7 cells for several reasons. Firstly, in these cells, CAMKII did not modify
intracellular calcium levels and this is further supported by the evidence that CAMKII can induce lasting increases in Nox5 activity in a cell free activity assay where the calcium concentration is fixed. Lastly, the loss of responsiveness of the S475A phosphonull mutant of Nox5 to CAMKII suggests that the mechanism by which CAMKII regulates Nox5 activity is not simply due to secondary changes in calcium. However, it is possible that in smooth muscle cells, which express both Nox5 and voltage gated calcium channels, that CAMKII could promote the elevation of intracellular calcium and this would serve to further increase Nox5 activity (Jay et al., 2008; Koch et al., 1990). The ability of CAMKII to regulate superoxide release from cells was also dependent on the presence of Nox5 as increased superoxide was not observed in control cells (without Nox5) or cells expressing Nox1.

In response to PMA, a recent study has ruled out CAMKII as a regulator of Nox5 activity based primarily on the lack of effect of the putative CAMKII inhibitor KN-92 on ROS production (Serrander et al., 2007). However, it should be noted that KN-92 does not effectively inhibit CAMKII and instead is often used an inactive structural analog of the active inhibitor, KN-93. In contrast, we found that superoxide release from BAEC expressing Nox5 and from COS-7 cells co-transfected with Nox5 and wt CAMKII was strongly inhibited by KN-93. PMA has also been shown to regulate the activity of voltage sensitive calcium channels (Hudmon et al., 2005) and thus it is possible that in cell types expressing these channels that PMA could indeed promote increased CAMKII activity through the
secondary elevation of calcium. However, this has not been shown to be important for Nox5 activity (Jagnandan et al., 2007).

As CAMKII is a calcium-calmodulin (Couchonnal and Anderson, 2008) regulated kinase, it is interesting that under resting conditions or in the absence of a calcium-transient that the activity of the WT kinase approached that of the constitutively active CAMKII. While a fraction of the CAMKII pool is thought to be active, this is unlikely to account for the high basal activity observed. One explanation for this is that ROS are a potent stimulus for increased activity of CAMKII (Howe et al., 2004; Trebak et al., 2010) and the elevated levels of ROS derived from the co-expression of Nox5 may be the cause of the increased activity of wt CAMKII. Indeed, a recent study has shown that CAMKII can be directly modified by the NADPH-oxidase dependent oxidation of two methionine residues which renders the enzyme constitutively active (Erickson et al., 2008).

The ability of ROS to stimulate CAMKII-activity has important functional consequences. Elevated ROS have been shown to potently upregulate the expression of eNOS in endothelial cells in a CAMKII-dependent manner (Cai et al., 2001). ROS-stimulated CAMKII also contributes to apoptosis of cardiac myocytes, impaired cardiac function and increased mortality following myocardial infarction (Erickson et al., 2008). More relevant to the current study, ROS in the form of H$_2$O$_2$ have been shown to positively regulate Nox5 activity via a c-Abl kinase, Ca$^{2+}$ mediated, redox dependent signaling pathway (El Jamali et al., 2008). However, it is not yet known whether CAMKs participate in this process. The ability of ROS to stimulate CAMKII activity and then secondarily increase
Nox5 activity also suggests the existence of a feed-forward mechanism that could lead to excessive ROS production via reciprocal stimulation.

The activities of Nox5 and CAMKII are strongly influenced by agonists that promote the mobilization of intracellular calcium and thus it is likely that both enzymes are co-activated when expressed in the same cell. Angiotensin II has been shown to stimulate CAMKII activity in vascular smooth muscle cells to promote hypertrophy (Li et al., 2010) and not surprisingly Angiotensin II is also a potent regulator of Nox5 (Montezano et al., 2010). Similarly, CAMKII and Nox5 are abundant proteins in sperm where they are important for motility and capacitation (Sabeur and Ball, 2007; Schlingmann et al., 2007). CAMKII plays an important role in cardiac myocyte excitation contraction coupling, calcium handling and apoptosis (Couchonnal and Anderson, 2008). It is not yet known if Nox5 is expressed in human cardiac myocytes although its expression has been documented in cardiac fibroblasts (Cucoranu et al., 2005).

In conclusion, the ability of CAMKII to regulate Nox5 activity may be significant in the regulation of ROS production that occurs downstream of calcium-mobilizing agonists such as angiotensin II. The ability of both proteins to reciprocally regulate the activity of each other could possibly contribute to excessive ROS production. Further studies are needed to identify the contribution of CAMKII to ROS production in diseases associated with excessive oxidative stress.
Authorship Contributions

Participated in research design: Pandey and Fulton

Conducted experiments: Pandey, Rafikov, Black, Gratton and Fulton.

Contributed new reagents or analytic tools: Pandey, Rafikov, Black

Performed data analysis: Pandey, Rafikov, Black, Gratton and Fulton.

Wrote or contributed to the writing of the manuscript: Pandey, Rafikov, Black, Gratton and Fulton
References


Footnotes
This work was supported in part by the Cardiovascular Discovery Institute at the Medical College of Georgia and grants from the National Institutes of Health [HL085827, HL092446] and the American Heart Association (EIA) to DF and the National Institutes of Health [HL60190 and HL67841] to SB.
Figure legend

Figure 1. Endogenous CAMKIIα regulates Nox5 activity. (A) Bovine aortic endothelial cells (BAEC) were transduced with Nox5 adenovirus (MOI 50) and incubated with vehicle (CON) or increasing concentrations (5µM and 10µM) of the CAMKII inhibitor, KN-93 for 30 min and superoxide release was measured using L-012 chemiluminescence. (B) Human Aortic Vascular Smooth Muscle Cells (HAVSMC) were transfected with 30nmol control (non-targeting) siRNA or siRNA selective for CAMKIIα and 48hrs later calcium-dependent ROS production was measured using L-012 chemiluminescence. Results are presented as mean± SEM, n=5-7. * p <0.05 versus vehicle or control siRNA.

Figure 2. Active CAMKIIα is sufficient for Nox5 activation. (A) COS-7 cells were co-transfected with HA-Nox5 and either control (lacZ) or WT-CAMKIIα cDNAs and basal superoxide release was measured. Cell lysates were immunoblotted for total Nox5 and CAMKIIα (lower panels). (B) COS-7 cells were co-transfected with HA-Nox5 and either control (lacZ) or WT-CAMKIIα in the presence and absence of KN-93 (10µM). Basal superoxide release was measured with L-012. Cell lysates were immunoblotted for total Nox5 (lower panel). Results are presented as mean± SEM, n=5. * p <0.05 versus vehicle or control lacZ. (C) COS-7 cells were co-transfected with HA-Nox5 and either control (lacZ), WT-CAMKIIα, dominant negative-CAMKIIα (DN, T305D) or constitutively active-CAMKIIα (CA, T286D). Basal superoxide release was measured using L012 and cell lysates were immunoblotted for total Nox5 (lower panel). Results are presented as mean± SEM (n=4-6), * p<0.05 vs. control(lacZ), # p<0.05 vs. WT CAMKIIα.

Figure 3. CAMKIIα directly increases Nox5 activity in isolated activity assays but does not modify intracellular calcium levels or the activity of other Nox enzymes. (A) Superoxide release from Nox5 in an isolated activity assay. Nox5 was extracted from detergent resistant microdomains of cells co-transfected with HA-Nox5 and a control (lacZ) or WT- CAMKIIα. Nox5 was incubated in a buffer containing100nM CaCl₂, 100µM FAD and superoxide initiated by injection of 100µM NADPH (indicated by the arrow). (B) Measurement of intracellular calcium in intact COS-7 cells expressing Nox5 and CAMKIIα using Fluo4-NW. Changes in fluorescence intensity were monitored with a 485/10nm excitation and a 520/10nm emission filter. Results are presented as mean± SEM, n=6, * P<0.05 vs. lacZ control. (C) COS-7 cells were co-transfected with Nox5 or Nox1, NoxA1, Nox01 with and WT-CAMKIIα or control DNA (lacZ) and basal superoxide was measured using L012 chemiluminescence. Results are presented as mean± SEM (n=5-6), * P<0.05 vs. control (lacZ).
Figure 4. CAMKIIα directly phosphorylates Nox5 on S498 and S494 in an in vitro kinase assay. (A) Nox5 was immunoprecipitated from COS-7 cells transduced with HA-Nox5 adenovirus and subject to an in vitro Kinase assay. Phosphorylated samples were immunoblotted for phosphorylated S490, T494 and S498 versus total Nox5. COS-7 cells were co-transfected with either (B) T494A or (C) S498A or (D) S490A, T494A, S498A triple mutants of HA-Nox5 together with WT-CAMKIIα or control DNA (lacZ). Basal superoxide was measured by L-012. Relative expression of Nox5 was detected by immunoblotting (lower panel). Results are presented as mean ± SEM (n=4-6), * P<0.05 vs. control (lacZ).

Figure 5. Mutation of S475A prevents CAMKIIα-dependent increases in Nox5 activity. (A) COS-7 cells were co-transfected with either wildtype (WT) HA-Nox5 or S475A or S659A or S502A mutants together with WT-CAMKIIα or control DNA (lacZ) and basal superoxide was measured with L-012. Cell lysates were immunoblotted for relative expression of HA-Nox5 (lower panels). Results are presented as mean ± SEM (n=4-5), * P<0.05 vs. lacZ control. (B) COS-7 cells were transfected with wildtype (WT) or S475A or S659A or S502A Nox5 and superoxide production was measured following stimulation with ionomycin (1µM). Results are presented as mean ± SEM (n=5-6), * P<0.05 vs. WT.

Figure 6. CAMKII-dependent phosphorylation of Nox5 on S475 in intact cells. (A) COS-7 cells were co-transfected with wild type (WT) Nox5 and S475A Nox5 together with CAMKIIα and cell lysates were probed for the relative expression of total HA-Nox5. (B) phosphoproteins in cell lysates were bound to phosphoprotein affinity columns and eluted proteins were probed for the expression of HA-Nox5. (C) Detection of phosphorylated amino acid modifications of Nox5 in COS-7 cells cotransfected with Nox5 and CAMKIIα. MS/MS spectra of parent ion m/z 1822.7 was obtained by MALDI-TOF-MS/MS fitted with peptide 464-SQGQWTNRLYES*FK-477 from the Nox5β sequence phosphorylated at S475.
Table I

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Phospho Peptide</th>
<th>Mascot Score</th>
<th>Sequest Score</th>
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</table>
| LYEpSFKA
DPLGR   | Ser 475         | 46           | 3.4           |
| KDPsITGL
QTR     | Ser 659         | 32           | 2.76          |
| SpSKGSE
ILLEK   | Ser 502         | n/d          | 0.84          |

Table 1. Identification of Novel CAMKIIα phosphorylation sites in Nox5. HA-Nox5β was immunoprecipitated from COS-7 cells transduced with HA-Nox5 adenovirus and subject to an in vitro kinase assay. Phosphorylated samples were run on SDS gel, silver stained and the band corresponding to the correct molecular weight of Nox5 was excised, trypsin digested and subject to analysis by mass spectrometry.
**Figure 1**

**A**

<table>
<thead>
<tr>
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<th>Superoxide Production RLU x (10^3)</th>
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<tbody>
<tr>
<td>Con</td>
<td>60 ± 5</td>
</tr>
<tr>
<td>5μM</td>
<td>40 ± 3</td>
</tr>
<tr>
<td>10μM</td>
<td>20 ± 2</td>
</tr>
</tbody>
</table>

**B**

Superoxide Production % of Control

- **IB: CAMKIIα**
  - Control siRNA
  - CAMKIIα siRNA

- **IB: GAPDH**
  - Control siRNA
  - CAMKIIα siRNA

* indicates significant difference.
**FIGURE 2**

A. Graph showing superoxide production with IB:HA-Nox5 and IB:CAMKIIIα. The values are represented as RLU x 10^3. Symbols * indicate statistical significance.

B. Graph showing superoxide production with HA-Nox5, CAMKIIIα, and KN-93. Symbols * and # indicate statistical significance.

C. Graph showing superoxide production with HA-Nox5, WTCAMKIIIα, Active CAMKIIIα, and DN CAMKIIIα. Symbols * and # indicate statistical significance.