Calcineurin A beta Regulates NADPH Oxidase (Nox) Expression and Activity via Nuclear Factor of Activated T Cells (NFAT) in Response to High Glucose*

Clintoria Williams, Emory University
Jennifer Gooch, Emory University

Journal Title: Journal of Biological Chemistry
Volume: Volume 289, Number 8
Publisher: American Society for Biochemistry and Molecular Biology | 2014-02-21, Pages 4896-4905
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1074/jbc.M113.514869
Permanent URL: https://pid.emory.edu/ark:/25593/rmgbt

Final published version: http://dx.doi.org/10.1074/jbc.M113.514869

Copyright information:
© 2014 by The American Society for Biochemistry and Molecular Biology, Inc.
Accessed January 24, 2020 1:20 AM EST
Calcineurin Aβ Regulates NADPH Oxidase (Nox) Expression and Activity via Nuclear Factor of Activated T Cells (NFAT) in Response to High Glucose*

Received for publication, September 3, 2013, and in revised form, October 18, 2013 Published, JBC Papers in Press, December 26, 2013, DOI 10.1074/jbc.M113.514869

Clintonia R. Williams†§ and Jennifer L. Gooch†§

From the †Atlanta Veterans Administration Medical Center, Atlanta, Georgia 30033 and the ‡Department of Medicine/Division of Nephrology, Emory University School of Medicine, Atlanta, Georgia 30322

Hypertrophy is an adaptive response that enables organs to appropriately meet increased functional demands. Previously, we reported that calcineurin (Cn) is required for glomerular and whole kidney hypertrophy in diabetic rodents (Gooch, J. L., Barnes, J. L., Garcia, S., and Abboud, H. E. (2003). Calcineurin is activated in diabetes and is required for glomerular hypertrophy and ECM accumulation. Am. J. Physiol. Renal Physiol. 284, F144–F154; Reddy, R. N., Knotts, T. L., Roberts, B. R., Molken-...
cells were grown at 37 °C in 5% CO2 in DMEM and F10 Hams growth medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. At 85% confluence, the culture medium was changed to serum-free medium. Cells were then exposed to medium containing normal glucose concentration (5.5 mmol/liter), high glucose concentration (12.5–25 mmol/liter), TGF-β, or angiotensin II for 10 min to 48 h. Experimental groups received fresh medium every 24 h. Select monolayers were treated with 1 μmol/liter diphenylhydantoin (DPI), 1 μmol/liter fulvene-5 (kind gift of Jack Arbiser, Emory University), 60 nmol/liter tacrolimus, 1–100 nM VIVIT peptide, or 0.01% vehicle (DMSO). In separate experiments, select monolayers were transfected overnight using Lipofectamine 2000 (Invitrogen) with small interfering RNA (siRNA) oligonucleotides against CnAβ (100 nmol/liter) or scrambled oligonucleotides (Ambion, Austin, TX), Nox2 or Nox4 cDNA (kind gifts of K. Griendling, Emory University), dominant negative NFAT, NFAT cDNA, or vector before 48 h of glucose exposure. Alternatively, cells were transfected overnight with an NFAT-luciferase reporter construct and then treated with serum-free or standard medium for 24 h.

**Measurement of Calcineurin Activity**

Calcineurin phosphatase activity was determined as described (18). Briefly, the calcineurin substrate peptide RII was synthesized with a phosphoserine at residue 15 and an amino terminus TAMRA fluorescent tag. In a 96-well plate, the labeled substrate was mixed in equal parts with reaction buffer and sample and allowed to incubate at 30 °C for 10 min. Supernatant from each well was then transferred to a 96-well plate coated with titanium oxide (TiO2) (Glygen, Baltimore, MD) followed by gentle shaking to allow binding of phosphorylated substrate.

**FIGURE 1.** The β isoform of calcineurin (CnAβ) is selectively activated by high glucose. A, Cn enzyme activity in WT kidney fibroblasts exposed to increasing amounts of glucose or calcium (Ca2+) for 10 min was determined by an in vitro Cn assay. Data shown are the mean ± S.E. (error bars) of triplicate reactions. *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with control. B, CnAα and CnAβ mRNA expression in WT, CnAα−/−, and CnAβ−/− kidney fibroblasts was examined by qRT-PCR. Data shown are the mean ± S.E. of 4–9 replicates/group. *, p < 0.05 compared with WT. C, Cn enzyme activity in WT, CnAα−/−, and CnAβ−/− kidney fibroblasts exposed to normal glucose (NG) or HG for 48 h was determined by an in vitro Cn assay. Data shown are the mean ± S.E. of 8 replicates/group. *, p < 0.05; **, p < 0.01 compared with NG; #, p < 0.05 compared with WT NG. D, Cn enzyme activity in WT, CnAα−/−, and CnAβ−/− kidney fibroblasts exposed to angiotensin II or TGF-β for 10 min was determined by in vitro Cn assay. Data shown are the mean ± S.E. of triplicate reactions. *, p < 0.05; ***, p < 0.001 compared with control. E and F, association of catalytic isoforms of Cn with calmodulin (CaM) was determined in WT, CnAα−/−, and CnAβ−/− kidney fibroblasts after 48 h of NG or HG exposure by a CaM pull-down assay. Relative amounts of CnAα and CnAβ bound to CaM were semiquantitated by densitometry. Data were normalized relative to WT NG and graphed. Data shown are the mean ± S.E. of four independent experiments. *, p < 0.05 compared with NG. #, p < 0.05 compared with WT NG.
Finally, supernatants containing unbound peptide were then moved transferred to a new 96-well plate, and the amount of dephosphorylated peptide was determined by fluorimetry at 540-nm excitation and 575-nm emission. Calcineurin activity was then determined by extrapolating the fluorescence of experimental samples from a standard curve of purified calcineurin (Sigma-Aldrich).

Calmodulin Pull-down Assay

Cells were lysed with TNESV lysis buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1% Nonidet P-40, 100 mM NaCl, 100 mM sodium orthovanadate, 100 μg/ml leupeptin, 20 μg/ml aprotonin, and 10−7 M phenylmethylsulfonyl (PMSF)), and 50 μg of protein was incubated overnight at 4 °C with calmodulin-conjugated agarose beads (Sigma-Aldrich). Beads were pelleted by centrifugation and resuspended in SDS-polyacrylamide gel loading buffer, and proteins were resolved by electrophoresis. Calcineurin isoforms were detected by Western blotting using antibodies that recognize the CnA and CnB isoforms (Millipore, Billerica, MA).

Determination of Cellular Hypertrophy

After a 48-h treatment, cells were incubated with 1 μM Hoechst in Krebs-Ringer phosphate buffer (KRPG; 145 mM NaCl, 5.7 mM KH2PO4, 4.86 mM KCl, 0.54 mM CaCl2, 1.22 mM MgSO4, and 5.5 mM glucose, pH 7.35) for 30 min at 37 °C to detect DNA content. Cells were washed with 1× phosphate-buffered saline (PBS), and fluorescence was measured at 350-nm excitation/460-nm emission. Finally, cells were lysed with TNESV buffer and the protein content was quantified using a bicinchoninic acid (BCA) assay (Pierce). Protein concentrations for each sample were normalized by respective Hoechst fluorescence intensity.

Analysis of CnA, CnB, and Nox Expression

Quantitative RT-PCR (qRT-PCR)—Total RNA was isolated from cells with TRIzol according to the manufacturer’s protocol (Invitrogen). cDNA was generated and amplified using One-Step SYBR Green (Bio-Rad). All data were normalized to the GAPDH content of the same sample, and mRNA expression was calculated using the ΔΔCt method (19).

Western Blot

Cells were harvested with trypsin-EDTA, pelleted, washed with 1× PBS, and lysed using TNESV buffer. In addition, snap-frozen whole kidney sections were homogenized with a Dounce Homogenizer in ice-cold TNESV buffer. 25 μg of protein was separated by 10% SDS-PAGE, and proteins were transferred to a PVDF membrane. 25 μg of protein was separated by 10% SDS-PAGE, and proteins were transferred to a PVDF membrane. The membrane was incubated in 1% bovine serum albumin-TBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) and then immunoblotted with appropriate dilutions of primary antibodies specific for Nox1 (Santa Cruz Biotechnology, Inc.), Nox2, Nox4 (Abcam, Cambridge, MA), or actin (Santa Cruz Biotechnology, Inc.). After washing, membranes were incubated with fluorescence-conjugated secondary antibody (LI-COR Biosciences, Lincoln, NE). Fluorescence detection was performed using an Odyssey imager (LI-COR Biosciences). Densitometry analyses were performed on 3–4 independent experiments using LI-COR Biosciences software.
**NFAT Mediates CnAβ Regulation of Nox2 and Nox4**

**Measurement of Reactive Oxygen Species**

H$_2$O$_2$ was measured by horseradish peroxidase-catalyzed oxidation of the nonfluorescent molecule N-acetyl-3,7-dihydroxyphenoxazine into the highly fluorescent molecule resorufin (Amplex Red Assay, Invitrogen). Fibroblasts or snap-frozen whole kidney sections were incubated in KRPG buffer containing 100 μl/ml Amplex Red and 0.2 units/ml horseradish peroxidase for 1 h at 37 °C. Resorufin fluorescence was measured at excitation and emission wavelengths of 540 and 590 nm, respectively. Sample fluorescence was compared with that generated by a H$_2$O$_2$ standard curve to calculate the concentrations of H$_2$O$_2$ released. H$_2$O$_2$ concentrations were normalized to cell tissue weight.

**Measurement of NFAT Activity**

Cells were transfected with 1 μg of NFAT-luciferase promoter construct described previously (20) as well as 100 ng of a Renilla promoter control plasmid. After treatment, cells were lysed using passive lysis buffer and centrifuged to pellet the debris, and then luciferase assay reagent (100 μl) was added to 20 μl of supernatant, and luminescence was measured for 10 s using an OptoComp Luminometer (MGM Instruments, Hamilton, CT).

**Statistical Analysis**

For all experiments, graphing and statistical analyses were performed using GraphPad software (Prism, San Diego, CA). Unless otherwise noted, statistical tests were two-way analysis of variance with Bonferroni’s post-test to detect differences between experimental groups. A value of $p < 0.05$ was considered statistically significant.

**RESULTS**

High glucose (HG) is an effective mechanism to induce hypertrophy in cultured renal cells. However, a direct effect of HG on Cn has not previously been examined. Renal fibroblasts were treated with increasing concentrations of HG for 10 min, and then Cn activity was examined using an *in vitro* enzyme assay. Fig. 1A shows that Cn was activated by HG in a dose-responsive fashion. 12.5 mM glucose was chosen for all subsequent experiments. Next, the effect of HG was examined on the two main isoforms of the catalytic subunit of Cn, CnAα and CnAβ, using renal fibroblast cell lines generated from WT, CnAα$^{-/-}$, or CnAβ$^{-/-}$ kidney cortices (described previously (9)). Loss of each isoform was verified by qRT-PCR (Fig. 1B). Next, each cell line was treated with 12.5 mM glucose, and Cn enzyme activity was examined. Fig. 1C shows that, although basal activity was lower compared with WT cells, HG increased activity in CnAα$^{-/-}$ cells. In contrast, basal activity was not different from WT in CnAβ$^{-/-}$ cells, and there was no change with HG. Similarly, induction was observed when WT or CnAα$^{-/-}$ cells were treated with angiotensin II or TGF-β, but no response was observed in CnAβ$^{-/-}$ cells (Fig. 1D). These data demonstrate that CnAβ and not CnAα was induced by hypertrophic signaling mechanisms. Activation of Cn requires...
that the catalytic subunit binds a regulatory, calcium-binding subunit (B) and calmodulin. To confirm a selective role for CnA\(_{\beta}/H9252\) in hypertrophic signaling, relative amounts of active CnA\(_{\beta}/H9251\) or CnA\(_{\beta}/H9252\) were determined by pulling down calmodulin from control or HG-treated cells and then immunoblotting for each isoform. In WT fibroblasts, CnA\(_{\beta}/H9251\) was the predominant isoform associated with calmodulin under basal conditions and did not change with HG treatment (Fig. 1E). CnA\(_{\beta}/H9251\) association with calmodulin in CnA\(_{\beta}/H9252\)/H11002 cells was comparable with WT but was absent in CnA\(_{\beta}/H9252\)/H11002 cells, as expected. In contrast, although there were very low levels of CnA\(_{\beta}/H9252\) bound to calmodulin under basal conditions, HG increased CnA\(_{\beta}/H9252\) binding (Fig. 1F). CnA\(_{\beta}/H9252\) binding to calmodulin was enhanced in CnA\(_{\beta}/H9251\)/H11002 cells and absent in CnA\(_{\beta}/H9252\)/H11002, as expected. Taken together, these data indicate that CnA\(_{\beta}\) is selectively induced by HG, whereas CnA\(_{\alpha}\) is constitutively active.

Next, the role of each Cn isoform in the induction of hypertrophy was examined. First, WT cells were treated with increasing concentrations of HG for 48 h, and the protein/DNA ratio was determined as a measure of hypertrophy. Fig. 2A shows that 12.5 mM HG was sufficient to induce hypertrophy, an amount comparable with maximal Cn activity induced by HG in Fig. 1A. Next, WT, CnA\(_{\alpha}/H9251\)/H11002, and CnA\(_{\beta}/H9252\)/H11002 cells were treated with HG, and the effect on the protein/DNA ratio was determined. Fig. 2B shows that HG induced hypertrophy in WT and CnA\(_{\alpha}/H9251\)/H11002 fibroblasts, whereas CnA\(_{\beta}/H9252\)/H11002 cells failed to respond. Interestingly, when CnA\(_{\alpha}/H9251\)/H11002 cells were pretreated with the calcineurin inhibitor tacrolimus (Tac), HG-stimulated hypertrophy was abolished (Fig. 2C). These data demonstrate that CnA\(_{\beta}\) mediates HG-mediated hypertrophy. To confirm a specific role for CnA\(_{\beta}\) in hypertrophy, WT cells were transfected with empty vector, CnA\(_{\alpha}\), or CnA\(_{\beta}\) cDNA under normal culture conditions, and then protein/DNA ratios were assessed after 72 h. Overexpression of CnA\(_{\beta}\) but not CnA\(_{\alpha}\) was sufficient to increase protein/DNA ratios (Fig. 2D).
Pharmacological inhibition of Cn has been shown to block hypertrophy \textit{in vitro} (4, 6) and \textit{in vivo} (1, 8). Similarly, blockade of chronic oxidative stress attributable to Nox inhibited hypertrophy \textit{in vitro} (4, 17) and \textit{in vivo} (14, 17). In particular, the Nox4 isoform has been implicated in diabetic kidney changes (12, 15, 21, 22). In Fig. 3 \textit{A}, the role of NADPH oxidases in the response to HG in renal fibroblasts was confirmed using a general inhibitor of NADPH oxidase activity, DPI, and a specific inhibitor of Nox2 and Nox4, Fulvene-5 (23). The data show that pretreatment with DPI or Fulvene-5 blocked HG-mediated hypertrophy in WT renal fibroblasts, confirming a central role for NADPH oxidase in the HG response. Next, generation of ROS was evaluated after 48 h of HG treatment. Fig. 3 \textit{B} shows that HG induced a significant increase in cellular ROS that is inhibited by DPI. Moreover, targeting of Nox2 and Nox4 with Fulvene-5 also attenuated ROS generation, indicating that Nox2/Nox4 induction is sufficient for HG-mediated oxidative stress. Therefore, we examined if CnA\textbeta{} and Nox cooperate in a common signaling pathway. First, ROS generation in response to 48 h of HG treatment was compared in WT and CnA\textbeta{}/H9252/\textbeta{}/H11002 cells. Fig. 3 \textit{C} shows that loss of CnA\textbeta{} reduced basal cellular ROS and blocked HG-mediated ROS generation. Consistent with these \textit{in vitro} findings, basal ROS was reduced in mouse kidneys (Fig. 3 \textit{D}). These results suggest that CnA\textbeta{} is also upstream of Nox \textit{in vivo}.

Expression of Nox isoforms was thus investigated in WT and CnA\textbeta{}/H11002 cells. In WT cells, HG increased Nox2 and Nox4 but not Nox1 expression (Fig. 4A), consistent with previous publi-
NFAT Mediates CnA\(\beta\) Regulation of Nox2 and Nox4

FIGURE 6. Nox2 and Nox4 mediate cellular hypertrophy. Relative expression of Nox2 (A) and Nox4 mRNA (B) was determined in CnA\(\beta^{-/-}\) kidney fibroblasts transfected with Nox2 or Nox4 cDNA. Data shown are the mean ± S.E. (error bars) of three independent experiments. *, \(p < 0.05\); ***, \(p < 0.001\) compared with vector. C, hypertrophy was measured in Nox2- or Nox4-transfected CnA\(\beta^{-/-}\) kidney fibroblasts by assessing the protein/DNA ratio. Data shown are the mean ± S.E. of three independent experiments normalized to WT NG vector. *, \(p < 0.05\); ***, \(p < 0.001\). D, ROS generation in Nox2- or Nox4-transfected WT kidney fibroblasts exposed to NG or HG for 48 h was examined by Amplex Red. Data shown are the mean ± S.E. of 8 replicates/group. ***, \(p < 0.001\) compared with NG. ALU, arbitrary light units.

CnA\(\beta^{-/-}\) cells demonstrated an impaired response to HG and attenuated expression of Nox2 and Nox4. To determine if Nox2/Nox4 are required for CnA\(\beta\)-mediated actions downstream of HG, both proteins were re-expressed in CnA\(\beta^{-/-}\) cells. Fig. 6, A and B, shows that Lipofectamine-mediated transfection of Nox2 or Nox4 cDNA increased basal mRNA expression in CnA\(\beta^{-/-}\) cells. Next, HG-mediated hypertrophy and ROS generation were examined. Fig. 6, C and D, shows that re-expression of Nox2 or Nox4 restored HG-mediated hypertrophy and ROS generation.

Cn activation by calcium results in dephosphorylation of substrates, including the NFAT family of transcription factors. Previously, NFAT has been shown to be activated in response to hypertrophic signaling in vitro (4, 6) and in vivo (1). Therefore, the role of NFAT in CnA\(\beta\)-mediated regulation of Nox2 and Nox4 was examined. First, activation of an NFAT-responsive luciferase reporter construct was examined in WT, CnA\(\alpha\) knockout, and CnA\(\beta^{-/-}\) cells. Fig. 7A shows that NFAT transcriptional activity can be stimulated in WT and CnA\(\alpha\) knockout, and CnA\(\beta^{-/-}\) cells but failed to respond in CnA\(\beta^{-/-}\) cells. Re-expression of CnA\(\alpha\) in CnA\(\beta^{-/-}\) fibroblasts (CnA\(\beta^{-/-}\)B) restored NFAT regulation. Next, the effect of a peptide inhibitor of NFAT, VIVIT, on hypertrophy and ROS generation was examined. Fig. 7B shows that VIVIT was sufficient to block HG-mediated ROS generation in a dose-responsive manner. Consistent with this, induction of Nox2 and Nox4 mRNA by HG was attenuated by VIVIT (Fig. 7C and D). Last, NFAT dominant negative overexpression in WT cells decreased Nox2 and Nox4 expression (Fig. 7E), whereas overexpression of NFAT increased expression (Fig. 7F). These findings demonstrate that CnA\(\beta\) regulates Nox expression through NFAT.

The results of these experiments indicated that CnA\(\beta\) is selectively activated by HG and dephosphorylates the transcription factor NFAT. Furthermore, CnA\(\beta\)/NFAT were required for HG-mediated expression of Nox2 and Nox4 and...
increased ROS generation (Fig. 8). Taken together, these data demonstrate a novel role of CnAα and NFAT in the regulation of Nox-mediated ROS generation. Because oxidative stress negatively alters the milieu of the intracellular environment of the kidney and adds to hyperglycemia-induced kidney damage, it is paramount that more studies investigate the mechanisms of this pathway.

**DISCUSSION**

This study builds on our previous work investigating isoform-specific functions of CnAα and CnAβ. Our findings indicate a primary role for CnAα in the development and function of the kidney (25, 26). This is consistent with data in this study showing that CnAα is probably the predominant isoform under basal conditions. In fact, the strong baseline association of CnAα with calmodulin and the lack of change in CnAα binding with stimuli suggest that CnAα is constitutively active. In contrast, although our previous data showed an absence of a significant defect in CnAβ−/− kidneys (25), diabetic hypertrophy is attenuated (2), suggesting that CnAβ plays a role in renal responses to stimuli. Enhanced CnAβ binding to calmodulin in response to HG and increased enzyme activity in cells expressing only CnAβ (Fig. 1) are consistent with CnAβ being an inducible enzyme.

Adding to the complexity of CnAα/CnAβ signaling, the data indicate that there may be crosstalk between the two isoforms. For example, Figs. 1B and 5A show that CnAα mRNA increases when CnAβ is absent or reduced. One possible mechanism is via altered expression of the endogenous calcineurin inhibitor CBP1/DSCR1, which is a transcriptional target of NFAT. Our data could support a model whereby loss of NFAT relieves repression of CnAα by CBP1/DSCR1. Isoform specificity of
NFAT Mediates CnAβ Regulation of Nox2 and Nox4

![Diagram]

In renal fibroblast cells, CnAα is constitutively active, whereas CnAβ is an inducible enzyme that is selectively activated by HG and other hypertrophic factors. CnAβ dephosphorylates NFAT, which translocates to the nucleus and (either directly or indirectly) induces the transcription of Nox2 and Nox4. Increased expression of Nox proteins leads to enhanced ROS generation and hypertrophy.

CBP1/DSCR1 has yet to be explored and is an important area for future study. Although these data suggest an intriguing mechanism of interaction between the two isoforms, we do not find evidence that elevated CnAα mRNA leads to increased basal activity. Fig. 1, C and E, does not show an increase in enzyme activity or calmodulin binding in the absence of CnAβ. Fig. 5B shows a slight increase in basal activity, but the change is not significant. This suggests that factors other than expression of the catalytic subunit may drive basal calcineurin activity. Because activation of the calcineurin holoenzyme requires interaction of the catalytic subunit with a regulatory subunit as well as calmodulin, it is possible that availability of the B regulatory subunit and/or calmodulin also influences basal activity.

In addition to further defining complementary roles of CnAα and CnAβ in the kidney, these data reveal that CnAβ plays a previously unknown role in regulation of Nox expression and function. Our data confirm the central role of Nox-mediated ROS generation in cellular changes with HG (Fig. 3). This increase in HG-mediated ROS is attenuated in CnAβ/−/− cells, and ROS generation is reduced in CnAβ/−/− kidneys, indicating that CnAβ is upstream of Nox function in vitro and in vivo. This hypothesis was confirmed by the data in Fig. 4 showing that loss of CnAβ is associated with decreased basal Nox2 and Nox4 and attenuation of HG induction of both enzymes. mRNA changes parallel expression of both proteins, supporting a transcriptional mechanism. Finally, selective knockdown of CnAβ in WT cells recapitulated the changes on Nox expression and HG response.

The data show that CnAβ is required for induction of Nox2 and Nox4 expression in response to HG in vitro. Although a number of signaling pathways downstream of CnAβ have been investigated in the setting of hypertrophy, this is, to our knowledge, the first time Nox has been linked to Cn action. Given the consistent finding that CnAβ is an integral player in hypertrophic signals in multiple tissues, it is possible that CnAβ regulates Nox expression and chronic oxidative stress in other organs as well. It is also intriguing to speculate that a lack of Nox2/Nox4 up-regulation is responsible for the blunted cardiac hypertrophy reported in 2002 (27) and whole kidney and glomerular hypertrophy reported more recently by our group in CnAβ/−/− mice (2).

In addition to establishing CnAβ as a novel upstream regulator of Nox2 and Nox4, this study also places the Cn substrate NFAT in the pathway. Overexpression and inhibition of NFAT modulates Nox2 and, to a lesser degree, Nox4 expression. Additional experiments are required to determine if NFAT acts directly on the Nox2 and/or Nox4 promoters or if NFAT acts indirectly either as a transcriptional co-factor or via regulation of another pathway. For example, NFAT is a member of the Rel transcription factor family along with NFκB. NFκB has been shown to regulate Nox4 (28). Moreover, Cn can dephosphorylate IκB (29), providing an additional avenue for cross-talk between the pathways. These data do not allow us to conclude if Nox4 and Nox2 are each regulated in parallel or if Nox2 is the target of CnAβ/NFAT regulation and that Nox2, in turn, regulates Nox4.

There are several interesting aspects of this pathway that warrant additional discussion and investigation. First, it is interesting that constitutive loss of CnAβ reduces basal Nox2 and Nox4 expression. Because Fig. 1 shows that CnAβ is not active in the absence of stimuli, it is possible that CnAα plays a role as an endogenous inhibitor of Nox2/4 expression. Supporting this model, siRNA to CnAβ attenuated HG-mediated up-regulation of Nox2/4 activity but had minimal effect on basal ROS. This model would also provide an explanation for the observation that calcineurin inhibitors increase Nox2 expression in vivo (30). If CnAα is constitutively active, the majority of effects from calcineurin inhibitor treatment will be due to down-regulation of CnAα. We have also found that CnAα/−/− cells have increased basal levels of Nox2 and Nox4.3 Taken together, these data confirm that CnAα and CnAβ may both be involved in aspects of regulation of Nox expression and ROS generation. Finally, the data show that constitutive loss of CnAβ also results in an increase in Nox1. This may represent a compensatory response to maintain cellular ROS. Additional experiments are necessary to identify the mechanism and significance of Nox1 up-regulation.

Finally, the current study was performed with spontaneously immortalized renal fibroblasts which is a potential limitation of the data. In vivo, proximal tubule epithelial cells are the site of greatest up-regulation of Nox4 in the diabetic kidney (17). In a limited set of experiments, we confirmed that Nox4 is also down-regulated in CnAβ/−/− tubule epithelial cells (data not shown). However, because cultured tubule epithelial cells do not undergo hypertrophy in response to HG in vitro, we chose to investigate the signaling pathway in renal fibroblasts. Nox4 expression and regulation have been reported in mesangial cells (31), podocytes (11, 32), and macula densa (13, 33), suggesting that although expression may be highest in the proximal tubule, regulation of Nox4 is widespread in the diabetic kidney. To confirm the relevance of our findings, we also show that ROS

3 C. R. Williams and J. L. Gooch, manuscript in preparation.
and Nox expression are reduced in CnAB−/− whole kidney lysates.

Although targeting of Nox4 is an area of therapeutic interest in the kidney, recent data showing that constitutive loss of Nox4 actually enhances kidney disease (24) highlights the need for additional study of the pathway. CnAB and NFAT are new players upstream of Nox that may offer an additional strategy for targeting Nox and hypertrophy in vivo.

Acknowledgments—We thank Dr. K. Griendling for reagents and for review of the manuscript and Drs. R. Price and H. Franch for helpful discussion of the data.

REFERENCES


Downloaded from http://www.jbc.org at Emory University on April 28, 2016
Calcineurin Aβ Regulates NADPH Oxidase (Nox) Expression and Activity via Nuclear Factor of Activated T Cells (NFAT) in Response to High Glucose
Clintoria R. Williams and Jennifer L. Gooch

doi: 10.1074/jbc.M113.514869 originally published online December 26, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M113.514869

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 33 references, 14 of which can be accessed free at http://www.jbc.org/content/289/8/4896.full.html#ref-list-1