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Mark Justin Jedrzejczak April 14, 2015

Calcineurin Activity in the Cardiac Left Ventricle of Transgenic AIDS mice that undergo experimental Myocardial Infarction: Experimental approaches to a new clinical problem

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An abstract of a
thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

Calcineurin Activity in the Cardiac Left Ventricle of Transgenic AIDS mice that undergo experimental Myocardial Infarction: Experimental approaches to a new clinical problem

By Mark Justin Jedrzejczak

In the era of antiretroviral therapy, the HIV population is living longer but becoming sick from diseases other than AIDS, so called HIV/AIDS non-AIDS diseases (HANA). Cardiovascular disease (CVD) is a HANA with increasing roles. The compensatory mechanisms for cardiac remodeling and hypertrophy are well characterized, but the role of HIV/AIDS in CVD is poorly understood. The protein phosphatase calcineurin (PPP3C) has been shown to activate nuclear factors of activated T-cells (NFATc) which initiate transcription of genes linked mechanistically to the development of left ventricle (LV) hypertrophy. Little is known about this canonical pathway in HIV/AIDS. To explore this relationship, transgenic mice (MSB) served as models of HIV/AIDS. MSB were subjected to myocardial infarct (MI) using ligation of a coronary artery ligation (CAL) to simulate the clinical event of cardiac ischemia. Alterations within the calcineurin-NFAT pathway were explored in this authentic HIV/AIDS models with MI. Expression of calcineurin and upstream signaling moieties were defined in murine LV samples by real-time PCR (qPCR) and analyzed statistically. Steady state polypeptide abundance was defined using SDS-PAGE Western blots while PPP3C phosphatase activity was assayed spectrophotometrically. qPCR data in MSB-CAL LVs showed decreased gene expression of relevant moieties in the calcineurin pathway including PI3KR1, PTEN, CAMK2D, CACNB2, CACNA2D1, CACNA1C, PKCB, CHP2, PPP3R1, PP3R2, PPP3CA, PPP3CB, PPP3CC, and RICTOR. Although polypeptide abundance remained unchanged, calcineurin activity in transgenic mice decreased twenty-four fold and activity in MSB-CAL mice exhibited a three-fold decrease. The consistent cellular changes in LVH signaling pathways have helped identify a previously unknown role of HIV/AIDS in MI.

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Finally to my parents, *Dziękuję Wam bardzo*. Thank You.

Table of Contents

Introduction

HIV/AIDS and Cardiovascular Disease

An estimated 34 million people worldwide live with the HIV infection, with 1.1 million documented to be living with the infection in the United States alone (WHO 2011 & CDC 2012). Antiretroviral drugs can slow the development of AIDS such that HIV/AIDS patients are now living long enough to become affected by aging and chronic illness such as cardiovascular disease (CVD). HIV infection and treatment have also been shown to increase the risk for CVD in men (Podzamczer 2013) and recently the same association was found in women (Womack, Chang et al. 2014). The role of HIV in CVD is still an emerging area of study and clinical concern (Phillips, Neaton et al. 2008).

Calcium signaling mechanisms

Cadiovascular diseases such as myocardial infarcts (MI) are characterized by ischemia, left ventricle hypertrophic compensation, dilation, and ultimate heart failure (Lim and Molkentin 1999). Known left ventricle hypertrophy (LVH) mechanisms are exquisitely regulated by intracellular calcium ($Ca^{2+)}$ levels which use L-type Ca^{2+} channels or LTCCs (Gomez, Ruiz-Hurtado et al. 2013). Cytosolic calcium levels are further influenced by two major cell signaling pathways: the PI3K-AKT and the phospholipase PTEN-PLC pathways (Fig. 1).

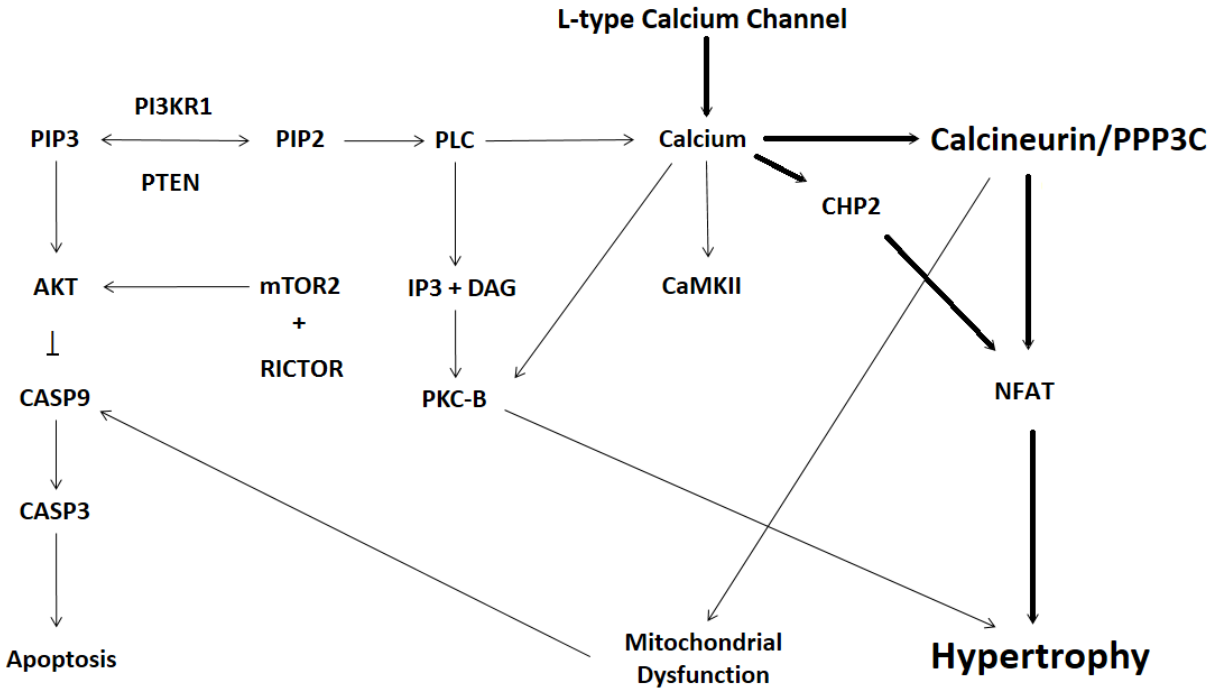


Figure 1. LTCC-mediated signaling pathways for cardiac hypertrophy along with key signaling mechanisms (AKT and PI3K/PTEN) which activate the protein phosphatase calcineurin (PPP3C) and downstream transcription factors (NFAT) responsible for hypertrophy.

Survival factors such as insulin growth factor (IGF-1) induce PI3K to initiate the process of phosphorylating phosphatidylinositol 4, 5-bisphosphate (PIP2) to PIP3. PIP3 then functions to anchor Akt, a major signal for cell growth and survival, which becomes phosphorylated at two sites: Thr308 by PDK1 (Alessi, James et al. 1997) and Ser473 by the mTOR-rictor complex (Wu, Chang et al. 2012). PIP3 is also converted into PIP2 by the phospholipase PTEN and subsequent phospholipase C (PLC) cleavage of PIP2 produces diacyl-glycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). The IP3/DAG pathway then activates protein kinase C (PKC) signaling and induces the release of calcium from the endoplasmic reticulum (ER) (Urena, Fernandez-Tenorio et al. 2013). Together, both LTCCs and the PI3K/PTEN pathway provide sources of calcium for downstream signaling.

Calmodulin-mediated Calcineurin-NFAT hypertrophy pathway

Calmodulin (CaM) is a key calcium sensor that detects changes in the level of intracellular Ca^{2+} ions (Shen, Lee et al. 2002). Only upon binding with calcium does CaM then bind and modulate the activity of other cellular proteins (Jurado, Chockalingam et al. 1999).

One binding partner of calmodulin is calcineurin (PPP3C or PP2B). PPP3C is a Ca^{2+} mediated calmodulin-dependent serine/threonine phosphatase that forms a heterodimer. It consists of a 61 kD catalytic subunit A and a 19 kD Ca^{2+} binding regulatory subunit B (Barford 1996). The A subunit has 3 isoforms encoded by the genes PP3CA, PP3CB, and PP3CC while the regulatory subunit B has two isoforms encoded by the genes PP3R1 and PP3R2. Additionally, there exists a protein product encoded by CHP2, calcineurin B homologous protein, which activates the phosphatase calcineurin subunit A. Both calcineurin and its homolog CHP2 function to mediate calcium-induced, biological signaling events that depend on the removal of phosphate groups for activation (Rusnak and Mertz 2000).

Specifically, calcineurin-dependent events trigger the activation of downstream nuclear factors of activated T cells (NFAT) in the regulation of cardiac remodeling and hypertrophy (Sanna, Kramer et al. 2002). Several studies (Rusnak and Mertz 2000, Molkentin 2004, and Sakuma and Yamaguchi 2010) suggest calcineurin plays an essential role in the regulation of cardiac hypertrophy, specifically through activation of NFATc1-4 transcription factors, which initiate the transcription of genes specific to cardiac development such as alpha-myosin heavy chain (α –MHC), alpha-actin, interleukin 6 (IL-6), insulin-like growth factor 1 (IGF-1), and myogenin.

Role of Calcineurin in HIV and Heart Failure

Calcineurin regulated LVH has yet to be explored in HIV/AIDS and MI, despite the well-known relationship between compensatory LVH and calcineurin activation in cardiac diseases. Based on data in other systems, it is hypothesized that genes and proteins within the L-type calcium-mediated calcineurin pathway will show decreased expression and that calcineurin phosphatase activity is decreased within MSB-CAL mice.

In order to better understand how this process may be affected in an HIV-1 transgenic mouse model (MSB), an established surgical technique of coronary artery ligation (CAL) can be used to generate an experimental myocardial infarct to study acute cardiac injury and remodeling *in vivo* (Virag and Lust 2011). Using a 2x2 factorial design, this study measures differential gene expression in the calcineurin-NFAT pathway, identifies changes in protein abundance, and assesses differences in calcineurin phosphatase activity in the MSB-CAL mouse model.

Materials and Methods

Mice: Cardiac left ventricle (LV) samples were taken from four different groups of heterozygous NL4-3 Δ gag/pol HIV-1 transgenic mice that are congenic to C567BL6/J or "black 6" mice: congenic with sham (null) surgery, congenic with the coronary artery ligation (CAL) surgery, transgenic MSB with sham surgery, and transgenic with CAL (MSB-CAL). All mice were treated in accordance with IACUC approved protocol in an AAALAC accredited vivarium at Emory University.

RNA Isolation: Cardiac LV RNA was isolated and purified from four mouse groups using a Qiagen RNeasy Fibrous Tissue Kit. Approximately 30 mg of cardiac tissue was homogenized at room temperature for 2 minutes at 20Hz (Retsch MM300 TissueLyser). Lysates were then eluted and purified following the RNeasy Fibrous Tissue Qiagen Kit protocol. Purified RNA was stored at -80°C.

cDNA Synthesis: Equal amounts of purified RNA were converted into cDNA using an Invitrogen SuperScript III First-Strand Synthesis as per manufacturer's protocol. After amplification in a thermal cycler, synthesis products were stored at -20°C.

LightCycler qPCR: Forward and reverse primer pairs were generated by Integrated DNA Technologies (see Appendix) for these genes of interest: (*PI3KR1, PTEN, CAMK2D, CACNB2, CACNA2D1, CACNA1C, PKCB, CHP2, PPP3R1, PP3R2, PPP3CA, PPP3CB, PPP3CC,* and *RICTOR).* The following two primers were designed as housekeeping genes: *ActB* and *Rn18s*. The cDNA synthesis product and SYBR Green I Master mix were run in duplicate on a 96-well plate. A standard Roche SYBR Green template protocol (see Appendix) was used for amplification (Roche LightCycler 480 System, Indianapolis). Amplification was analyzed and quantitated in GraphPad Prism 5 software.

Western Blotting: MSB-CAL tissue samples were lysed in RIPA and proteinase inhibitor cocktail buffer (1:100). Using a Misonix XL-2000 ultrasonic liquid processor, lysates were sonicated and spun down at 9,700 RPM for 15 minutes at 4°C (Beckman Coulter Allegra 25R Centrifuge). The protein supernatant was stored at -80°C. Protein concentrations were then quantified by Bradford assay in a Molecular Devices Spectra Max 190 spectrometer using SoftMax Pro 4.8 software.

Samples were diluted to a final 30ug/mL concentration of protein. Proteins were then resolved by precast SDS-PAGE (Bio-Rad), transferred to PVDF membrane (Thermo Scientific), and probed with calcineurin A (NBP1-33041), calcineurin B (NBP2-15664), NFATc3 (SC- 8405), NFATc4 (SC-13036), CHP2 (NBP1-68411), pan Akt (Cell Signaling Technology-C67E7), phospho-Akt Thr308 (CST-13038), phospho-Akt Ser473 (CST-4060), RICTOR (NB100-56427SS), and β-actin (Sigma 5316) antibodies. Developed film blots were then quantified using ImageJ and GraphPad Prism 5 software.

Calcineurin Activity Assay: Cardiac LV tissue samples suspended in RIPA lysis buffer were desalted and concentrated by centrifugation with Amicon Ultra 0.5mL Centrifugal 3K Filters. Sample concentrate was mixed with Green Assay Reagent (Abcam) to ensure adequate desalting. Enzyme activity was determined using a Cellular Calcineurin Phosphatase Activity Assay Kit (ab139464) and the procedure followed was the protocol as provided by the manufacturer. Fluorescence created by the formation of a chromogenic complex with malachite green, ammonium molybdate, and phosphate ions was read at OD_{620nm} and results were quantified with GraphPad Prism 5 software.

Results

Identification and validation of changes in transcription: In an attempt to identify and characterize the effects of MI in transgenic mice, a 2x2 factor approach was taken to separate MI as one variable and transgenesis as the other. Cardiac LV tissue was quantified at the transcriptional, translational, and enzymatic levels. These results were then observed by two-way ANOVA to determine how the MSB-CAL models interacted.

To define any changes at the level of transcription, fourteen genes of interest were chosen based on previous microarray analyses. These include genes, homologous between humans and the mouse model, that are directly involved in calcium signaling (LTCC) and in the calcineurin-NFAT hypertrophy pathway. To identify and determine changes in the gene expression of proteins in the calcineurin-mediated pathway responsible for LVH a total of 16 genes (Table 1) were amplified using real-time PCR. Two housekeeping genes were assayed as controls.

Transcription levels for genes within the LTCC mediated calcineurin pathway were determined using purified RNA isolated from cardiac left ventricle tissue lysates. After obtaining DNA via kit-based RNA to cDNA conversion, changes in gene transcription levels were identified and quantified by real-time polymerase chain reaction. The fold change in gene expression for the calcium channel voltage-dependent subunits (Fig. 2A) was found to be decreased in both HIV-1 transgenic (MSB) and wild type mice with coronary artery ligation (CAL) for CACNA2D1 as compared to sham surgery. CACNA1C and CACNB2 expression in CAL mice decreased independently of transgene. Furthermore, MSB-CAL mice had the lowest fold change for two of the three tested calcium channel genes.

Three gene targets related to cell growth (Fig. 2B) were analyzed for variation. Expression of PTEN had the second largest reduction in fold change: MSB-CAL differed from wild type sham by about 90%. Expression of RICTOR decreased by more than half between sham and CAL mice, and MSB-CAL mice had nearly a four-fold reduction. PI3KR1 had over 100 percent decreased expression between wild type-CAL and MSB-CAL mice.

Protein kinase C beta (PKCB) expression consistently decreased by two-fold in HIV-1 transgenic mice (Fig. 2C). Calmodulin-dependent kinase (CAMK2D) mRNA expression also decreased by half in both transgenic and CAL mice.

Calcineurin B homologous protein 2 expression was consistently decreased by half in the transgenic groups, independent of surgery (Fig. 2D). Four of the five gene isoforms of calcineurin were consistently decreased in the MSB-CAL model. These isoforms included all three catalytic subunits and one regulatory subunit. The MSB-CAL model had the lowest expression in all five calcineurin isoforms.

Calcineurin-NFAT protein expression by western blot

To identify and demonstrate changes in protein translation in the calcium/calmodulin dependent calcineurin pathway, a total of 10 antibodies were used to probe for protein products of the genes AKT1 (total and p473), calcineurin B homologous protein (CHP2), calcineurin subunits A and B, downstream transcription factors NFATc3 and NFATc4, and mTOR component RICTOR. The five blots (CHP2, CAL A, both NFATc3/4, and RICTOR) that failed to produce results occurred due to a combination of nonspecific antibody binding and overexposure. The remaining half, including the actin control, yielded results adequate enough for further image quantification. Three of these groups, however, (total AKT1, phosphorylated total AKT1, and p473) had too small of a sample size (n=2) to warrant complete data.

The protein expression of calcineurin subunit B protein, with sets of n=3 and n=5 (Fig. 4), had no significant changes in either the column or row factor. Transgenic mice exhibited a trend of lower calcineurin B expression than the wild type counterparts in their respective surgery groups (Fig 5.) The small sample size and variable SEM levels, however, precluded any meaningful statistical interactions.

Calcineurin Activity Assay

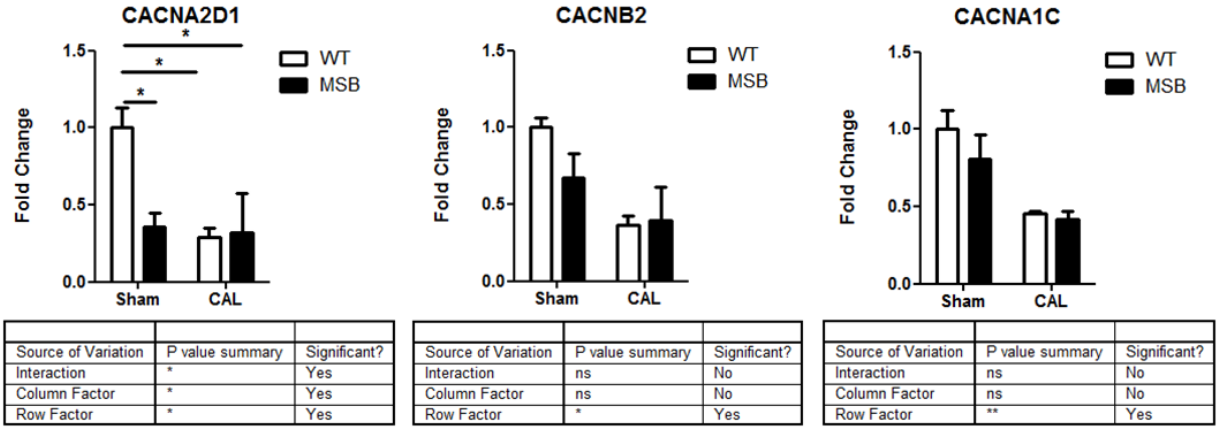
Calcineurin protein phosphatase activity (PPP3) was assayed to complete the characterization of the effects of embryonic non-virulent HIV-1 infection in mice (MSB) with cardiac ischemia-inducing coronary artery ligation (CAL). Whole protein lysates of cardiac left ventricle (LV) tissue from each of the 4 mouse groups (wild type with or without CAL and transgene with or without CAL) were desalted of free-floating phosphates. This helped reduce the level of background noise as the Abcam Calcineurin Phosphatase Activity Assay functions by highlighting released phosphates through fluorescence. Acceptable levels of background phosphates were achieved after 5 spin cycles (data not shown).

Mice with the HIV-1 transgene plus sham surgery had a remarkably decreased (Fig. 6) PPP3C activity; 24 times lower relative to wild type-sham mice. Calcineurin activity in mice with the HIV-1 transgene and CAL surgery (MSB-CAL) was three times lower than control for transgene and myocardial ischemia. Independent of surgery, MSB mice had half of the calcineurin activity that wild type mice exhibited. Consistent with gene expression, calcineurin activity was overall the lowest in the MSB-CAL model.

Figures and Tables

Table 1. List of genes converted from mRNA and analyzed as cDNA by qPCR, with protein products and function listed for reference. *Used as reference genes for standardizing fold change.

Gene	**Protein Product**	**Protein Function**
CACNA1C	Calcium channel alpha 1C subunit	Mediates influx of Ca^{2+} ions
CACNA2D1	Calcium channel alpha 2/delta 1 subunit	Mediates influx of Ca^{2+} ions
CACNB2	Calcium channel beta 2 subunit	Mediates influx of Ca^{2+} ions
PTEN	Protein and lipid phosphatase	Tumor suppressor
PI3KR1	Phospholipid kinase	Cell survival and proliferation
RICTOR	Subunit of mTORC2	Regulates cell growth and survival
PKCB	Protein Ser/Thr kinase	Hypertrophy signaling
CHP2	Calcineurin B Homologous Protein	Stimulates phosphatase activity
CAMK2D	Calcium/calmodulin-dependent delta subunit	Calcium signaling kinase
PPP3R1	Calcineurin subunit B, alpha isoform	Senses calcium and activates calcineurin
PPP3R2	Calcineurin subunit B, beta isoform	Senses calcium and activates calcineurin
PPP3CA	Calcineurin alpha isozyme	Calcineurin catalytic subunit
PPP3CB	Calcineurin beta isozyme	Calcineurin catalytic subunit
PPP3CC	Calcineurin gamma isozyme	Calcineurin catalytic subunit
ACTB*	Beta actin	Cell motility, structure, integrity
Rn18s*	Mouse Ribosomal RNA	RNA



Source of Variation	P value summary	Significant?
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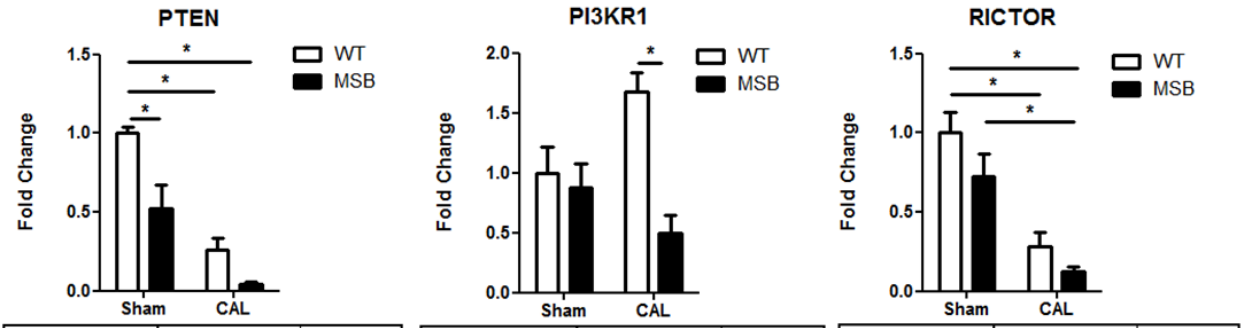
Column Factor	*	Yes
Row Factor	*	Yes

Source of Variation	P value summary	Significant?
Interaction	ns	No
Column Factor	ns	No
Row Factor	*	Yes

Source of Variation	P value summary	Significant?
Interaction	ns	No
Column Factor	ns	No
Row Factor	**	Yes

Figure 2A. L-type Ca^{2+} Channel gene fold change in MSB-CAL model (+SEM) relative to housekeeping genes ACTB and Rn18s compared with a significance of $P<0.05$ by two-way ANOVA.



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Row Factor	***	Yes

Source of Variation	P value summary	Significant?
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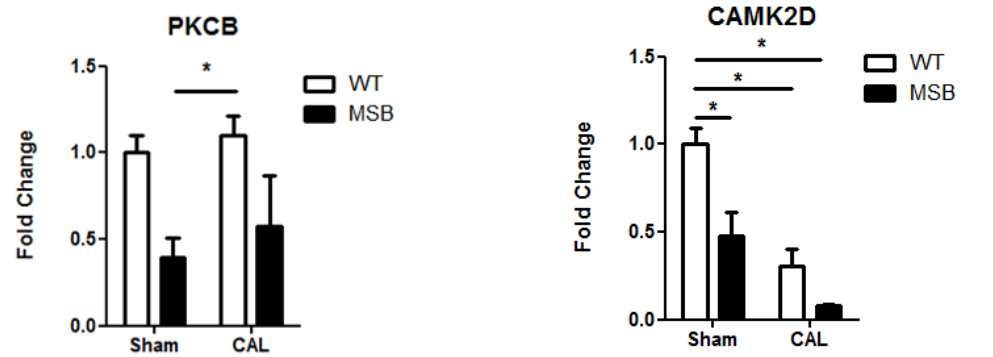
Column Factor	*	Yes
Row Factor	ns	No

Source of Variation	P value summary	Significant?
Interaction	ns	No
Column Factor	ns	No
Row Factor	***	Yes

Figure 2B. Cell survival and growth gene fold change in MSB-CAL model (+SEM) relative to housekeeping genes ACTB and Rn18s compared with a significance of P<0.05 by two-way ANOVA.



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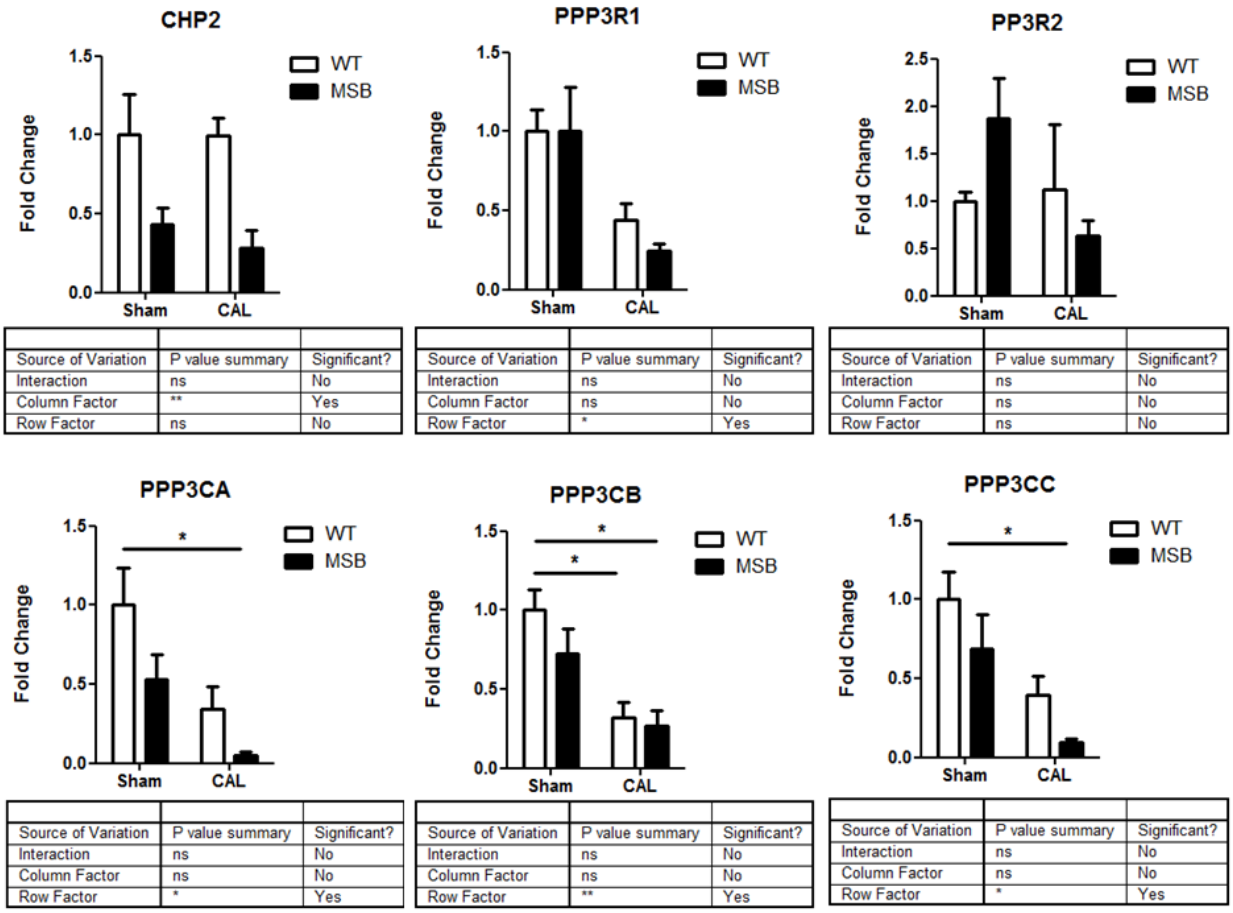
Column Factor	**	Yes
Row Factor	ns	No

Source of Variation	P value summary	Significant?
Interaction	ns	No
Column Factor	*	Yes
Row Factor	**	Yes

Figure 2C. LVH signaling mean gene fold change from MSB-CAL model (+SEM) relative to housekeeping genes ACTB and Rn18s compared with a significance of P<0.05 by two-way ANOVA.



Source of Variation	P value summary	Significant?
Interaction	ns	No
Column Factor	**	Yes
Row Factor	ns	No

Source of Variation	P value summary	Significant?
Interaction	ns	No
Column Factor	ns	No
Row Factor	*	Yes

Source of Variation	P value summary	Significant?
Interaction	ns	No
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Row Factor	ns	No

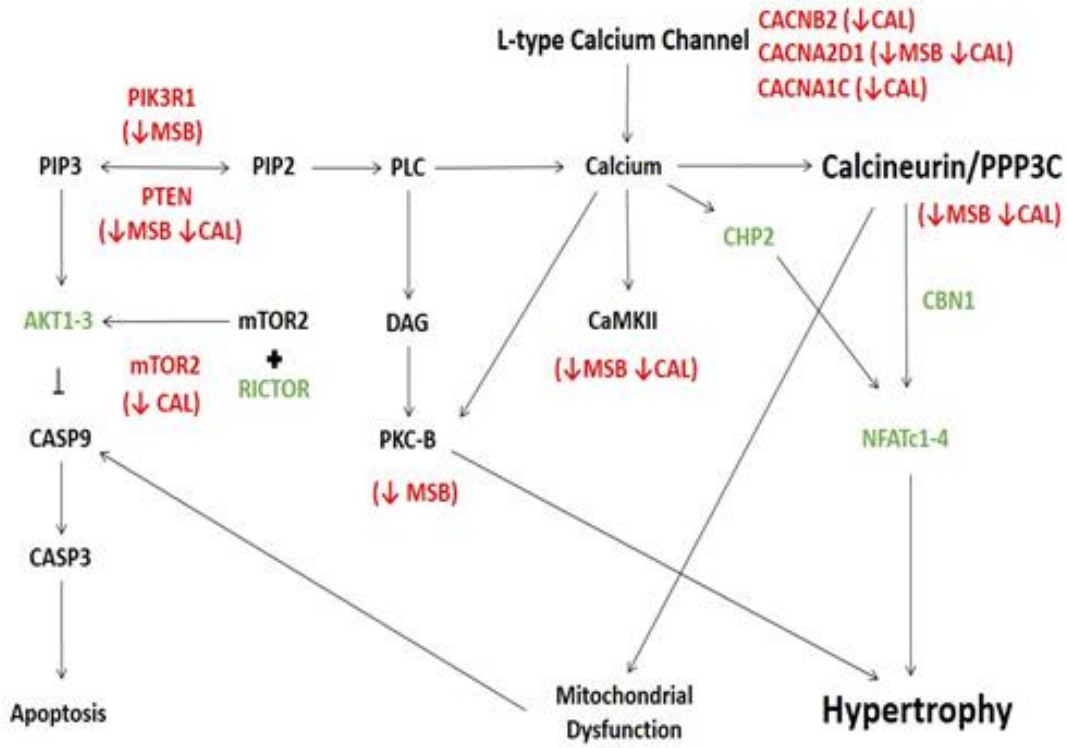
Source of Variation	P value summary	Significant?
Interaction	ns	No
Column Factor	ns	No
Row Factor	*	Yes

Source of Variation	P value summary	Significant?
Interaction	ns	No
Column Factor	ns	No
Row Factor	**	Yes

Source of Variation	P value summary	Significant?
Interaction	ns	No
Column Factor	ns	No
Row Factor	*	Yes

Figure 2D. Calcineurin B homologous protein 2 and five calcineurin (PPP3) isoform mean gene fold change (+SEM) of mRNA from MSB-CAL cardiac LV tissue Each set (n=5) was quantified relative to reference genes *ACTB* and *Rn18s*. Data were compared with a significance of P<0.05 by two-way ANOVA.

Figure 3. LTCC & Calcineurin-NFAT pathway: changes in genes expression. Red coded genes represent the moieties with decreased transcription using qPCR data from MSB-CAL mice. Green coded were targets for protein abundance, however theses did not produce adequate data (see Results).



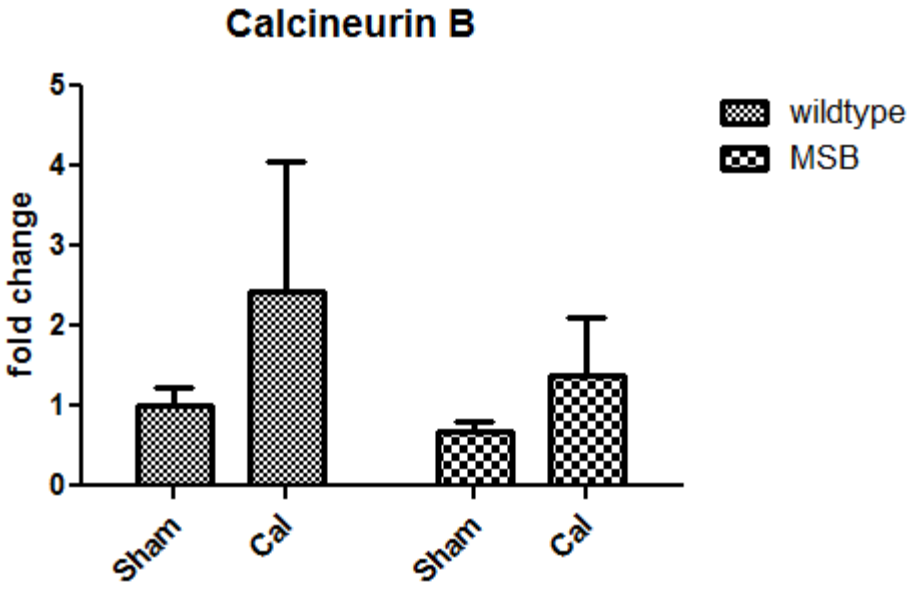


Figure 4. Fold change (+SEM) of calcineurin subunit B protein expression normalized to actin in cardiac left ventricle tissue from MSB-CAL mice. Expression quantified as area under the curve in ImageJ software. Data were compared with a significance of $P<0.05$ by a two-way ANOVA (sham/Cal sets n=3/5 respectively).

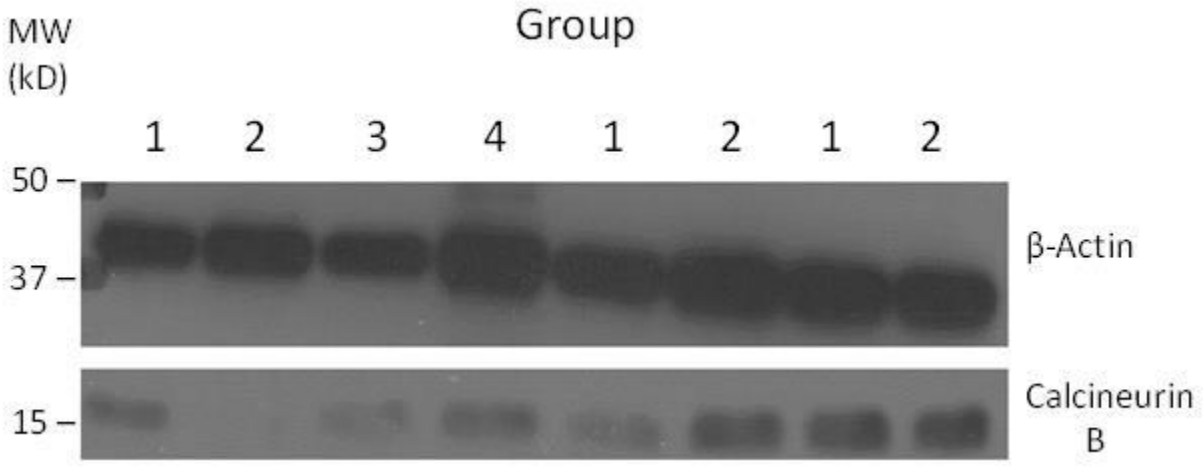


Figure 5. SDS polypeptide abundance of Calcineurin subunit B (19 kD) and β-Actin as loading control (42 kD). Group 1 corresponds to wildtype-sham, 2 is wildtype-CAL, 3 is MSB-sham, and group 4 is MSB-CAL.

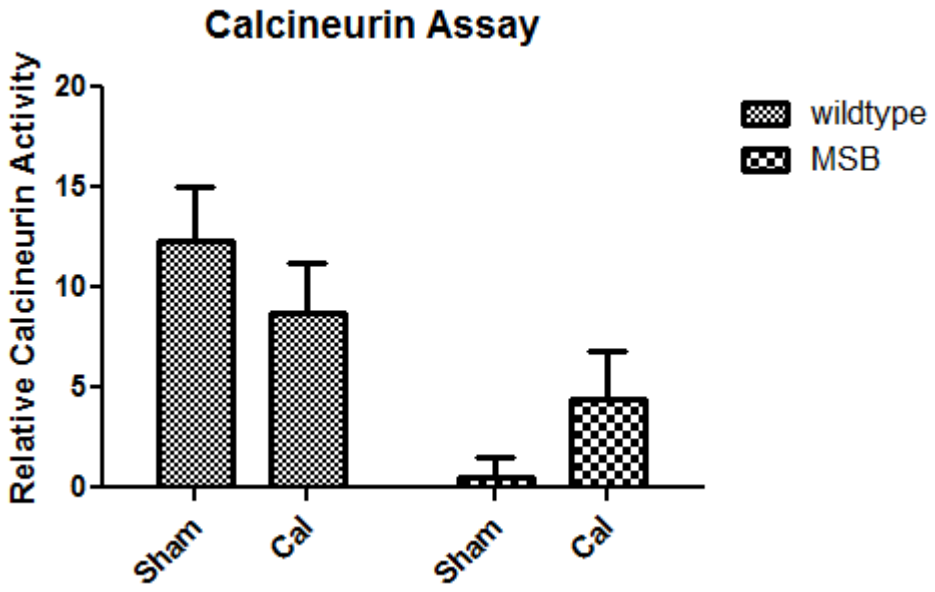


Figure 6. Calcineurin protein phosphatase (PPP3) activity (+SEM) normalized relative to total protein concentration of cardiac LV tissue from MSB-CAL mice. Data sets (total n=16) were analyzed with a significance of $P<0.05$ by two-way ANVOA.

Discussion

Previous studies have shown that L-type calcium channels (LTCCs) play a critical role in regulating calcium in cardiac myocytes (Mukherjee and Spinale 1998, Viola, Macdonald et al. 2009). Changes in cytosolic calcium induce growth-promoting signals, indicating that LTCCs function as pathways to trigger LVH (Gao, Wang et al. 2012).

It has been shown that calcium-induced calcineurin phosphatase activity functions to initiate LVH through the activation of a family of NFAT transcription factors, which in turn elicit a hypertrophic growth response (Tongers, Fiedler et al. 2004). It is also known that the calcineurin-NFAT pathway is one of two distinct modes of cardiac remodeling found in mice, the second being via MEK1-ERK1/2, which augments NFAT activity independently of calcineurin (Sanna, Bueno et al. 2005). What has previously been unknown is how the calcineurin-NFAT hypertrophy response might change in an HIV-1 model.

In this study, we have found the first evidence to suggest that HIV-1 transgenic mice with myocardial ischemia have reduced transcription of LTCC gene CACNA2D1, and that transcription of the LTCC genes CACNA1C and CACNB2 was reduced in CAL mice, independently of transgene. We also found a remarkably consistent decrease in gene expression of CAMK2D and five of the six isoforms of calcineurin, each of which plays a regulatory or catalytic role in the activation of NFATs, suggesting a tightly regulated transgenic influence (Hogan, Chen et al. 2003).

Activation of either PI3K through the IGF-1 pathway or Akt through the mTOR/Rictor complex is known to lead to a positive effect on LVH (Glass 2003). Compensatory left ventricle hypertrophy (LVH) initially helps protect cardiac function, however sustained stress results in dilation and heart failure. Just recently, one group found evidence confirming the concept that long-term Akt activation in mice leads to cellular and metabolic alterations characteristic of pathological LVH (Wende, O'Neill et al. 2015).

In this study, we have found cell growth and hypertrophy signals (LTCCS, RICTOR, PTEN, and PIK3R1) exhibit consistently lower gene expression in MSB-CAL mice. The crucial calmodulin and calcineurin isoforms, necessary for NFAT-induced hypertrophy, also have decreased mRNA levels. In terms of protein abundance, not enough data were found to provide conclusive results. Future studies will need to quantify relevant protein abundance in this pathway to validate if protein levels change proportionally with transcription (Vogel and Marcotte 2012). We also found that calcineurin phosphatase activity was attenuated in both the transgenic and MSB-CAL cohorts, with a statistical significance in the transgenic sham model. Interestingly, the MSB-sham model had lower activity than the wildtype-CAL model, which might be due to a specific mechanism caused by the HIV infection alone. In general, the evidence points to a trend of disruption in the calcineurin-NFAT hypertrophy signaling pathway in HIV-1 transgenic mice with cardiac ischemia.

These two consistent changes at the transcription and enzyme level suggest that calcineurin-NFAT genes and proteins are strongly regulated in the HIV-1 model, possibly by upstream and downstream effectors. Distinct NFAT-controlled LVH pathways, such as the MEK1-ERK1/2 pathway, might entirely compensate for the loss of the calcineurin-NFAT route

(Sanna, Brandt et al. 2005). Additionally, it would be valuable to investigate known partners that post-translationally modify calcineurin such as modulatory calcineurin-interacting proteins 1 & 2 (MCIP1 & 2), the slight increase of which can reportedly inhibit calcineurin-NFAT signaling (Sanna, Brandt et al. 2006). The elucidation of specific mechanisms by which these changes occur would also prove highly useful in understanding the contribution of HIV in heart disease.

Though the primary mechanism is yet unknown, this study proposes that the source of these cellular alterations is the HIV infection. The data suggest that cardiac left ventricle tissue, subjected to an acute myocardial infarct in the HIV-1 transgene mouse, exhibits decreased cellular signaling, reduced genetic expression, and attenuated PPP3C activity within the calcineurin-NFAT pathway responsible for hypertrophy, a hallmark of heart failure and CVD. Both male and female HIV/AIDS patients have been shown to be at an increased risk of CVD due to the infection, highlighting the importance of understanding HANA diseases. Research in this field, using the MSB-CAL mouse model, will help illuminate a previously unreported role of HIV in a HANA disease pathway and it presents the opportunity for future studies of clinical relevance.

Appendix

qPCR Primer Sequences from Integrated DNA Technologies

CACNA1C

Forward: (5’-AGTGAGACTAATCCAGCTGAACA-3’)

Reverse: (5’- AGATGCGGGAGTTCTCCTCT-3’)

CACNB2 F: (5’- ATC TCG AGG GAA ATC TCA AGC AA-3’)

R: (5’- TGA TTC CTG CGG AGG ACA TTG G-3’)

CACNA2D1

F: (5’- TTT ACA CTC TGT CCC AAT GGC T-3’)

R: (5’- GAG ATT TGG GGT TCT TTG GCT -3’)

PI3KR1

F: (5’- CCT TAA ATG GTG AGC ACG GAG -3’)

R: (5’- ACA CAC CCC AGC CAA TCA AG -3’

PTEN

F: (5’-TGG ATT CGA CTT AGA CTT GAC CT -3’)

R: (5’-ATG TCT CTC AGC ACA TAG ATT GT -3’)

RICTOR

F: (5’-ACT GAC GCC AAG CAG GTT TAT -3’)

R: (5’- GCG CTG GAG GGT ATT GTG AT -3’)

PKCB

F: (5’- CAA AAG CTT GTG GGC GAA AC-3’)

R: (5’- CCT GGT CAG GAG GTG TTA GG-3’)

Chp2

F: (5’- TTC CTA AGC CGC CTA GAC CT-3’)

R: (5’- GTC TCT GAC TTC CGT TGG GG-3’)

PP3CB

F: (5’- GAG AAA AAG TGA CAG AAA TGT TGG T-3’)

R: (5’- CTG GTC TTC ACC TTC TGT CAT-3’)

PP3CA

F: (5’- CAA AGC GCT ACT GTT GAG GCT A-3’)

R (5’- GCC TCC TCG AAG CTA GTG AT-3’)

PP3CC

F: (5’- GAG ACT GCC AAA CAA GAA GCC-3’)

R (5’- ACT TCG GAT CCT GTG TGC AA-3’)

PP3R1

F (5’- TGT GCTCAC ACT TTG ATG CTG-3’)

R (5’- TCC ACG CTC AAA GAA CCA GA-3’)

PP3R2

F (5’- GCG ATG GCC GGA TAT CCT TT-3’)

R (5’- CCG TGT TCT ACA AAC ACG ACC-3’)

CAMK2D

F (5’- GGG CGC CAT CTT GAC AAC TA-3’)

R (5’- CTC AGT TGA CTC CTT TAC CCC AT-3’)

Rn18s

F (5’- TGCAAAGCTGAAACTTAAAGGA-3’)

R: (5’- CCACGGAATCGAGAAAGAGCTA-3’)

Actb

F: (5’- GCT TCT TTG CAG CTC CTT-3’)

R: (5’- GAC CAG CGC AGC GAT A-3’)

qPCR protocol

Amplification: 45 cycles of 95^{o}C for 10 seconds, 54^{o}C for 15 seconds, and 72^{o}C for 23 seconds per cycle.

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