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Chronic Binge Alcohol-Induced Dysregulation of Mitochondrial-Related Genes in Skeletal Muscle of Simian Immunodeficiency Virus-Infected Rhesus Macaques at End-Stage Disease

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Abstract

Aims: Alcohol use disorders are more prevalent in HIV patients than the general population. Both chronic alcohol consumption and HIV infection have been linked to mitochondrial dysregulation; and this is considered an important mechanism in the pathogenesis of muscle myopathy. This study investigated if chronic binge alcohol (CBA) administration impairs the expression of genes involved in mitochondrial homeostasis in SIV-infected macaques.

Methods: Male rhesus macaques were administered daily CBA (to achieve peak blood alcohol concentrations of 50–60 mM within 2 h after start of infusion) or sucrose (SUC) intragastrically 3 months prior to intravenous SIV_{mac251} inoculation and continued until macaques met criteria for end-stage disease. Skeletal muscle (SKM) samples were obtained at necropsy. Muscle samples were obtained from a cohort of healthy uninfected macaque controls and used for comparison of analyzed variables. Total RNA was extracted and gene expression was analyzed by quantitative polymerase chain reaction.

Results: The relative expression of peroxisome proliferator-activated receptor gamma coactivator-1 beta (PGC-1β) was significantly decreased in the SKM of CBA/simian immunodeficiency virus (SIV) macaques compared to uninfected controls ($P < 0.05$). SIV infection resulted in a significant upregulation ($P < 0.05$) of mitophagy-related gene expression, which was prevented by CBA. CBA suppressed expression of anti-apoptotic genes and increased expression of pro-apoptotic genes ($P < 0.05$).

Conclusions: These findings suggest that SIV infection disrupts mitochondrial homeostasis and when combined with CBA, results in differential expression of genes involved in apoptotic signaling. We speculate that impaired mitochondrial homeostasis may contribute to the underlying pathophysiology of alcoholic and HIV/AIDS associated myopathy.

Short summary: This study investigated if CBA administration dysregulates gene expression associated with mitochondrial homeostasis in the SKM of SIV-infected macaques. The results suggest that SIV infection disrupts mitochondrial homeostasis and when combined with CBA, results in differential expression of genes involved in apoptotic signaling.

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INTRODUCTION

An estimated 1 million persons are currently living with HIV/AIDS (PLWHA) in the USA (CDC, 2013) and ~35% of them meet the criteria for having an alcohol use disorder (AUD) (Lefevre *et al.*, 1995; Bing *et al.*, 2001; Conigliaro *et al.*, 2003; Galvan *et al.*, 2003; Justice *et al.*, 2006). The prevalence of AUD in HIV patients is greater than the prevalence of AUD in the general population (Galvan *et al.*, 2003; Grant *et al.*, 2004). Effective antiretroviral therapy (ART) drug regimens have significantly decreased mortality of HIV-infected individuals, making HIV infection a chronic illness (Walensky *et al.*, 2006; HIV-CAUSAL Collaboration *et al.*, 2010; Nakagawa *et al.*, 2012; Deeks *et al.*, 2013; Fauci and Marston, 2013.) However, prolonged survival has been associated with increased incidence of comorbidities including skeletal muscle (SKM) myopathy and mitochondrial dysfunction (Maagaard and Kvale, 2009; Huang *et al.*, 2012; Keithley and Swanson, 2013). Decreased muscle mass and function remains a strong and consistent predictor of mortality among PLWHA and the frequency of low muscle mass is seen at a much younger age among PLWHA compared to the general population (Tang *et al.*, 2005; Richert *et al.*, 2011). This affects the quality of life and increases the incidence of mortality among these patients. Independently, chronic heavy alcohol consumption and HIV infection both result in significant SKM derangements such as atrophy, weakness and dysfunction (Preedy and Peters, 1994; Scott *et al.*, 2007; Vary and Lang, 2008; Clary *et al.*, 2011; Richert *et al.*, 2011).

Previous studies from our laboratory have demonstrated that chronic binge alcohol (CBA) administration exacerbates metabolic dysfunction in non-ART treated simian immunodeficiency virus (SIV)-infected macaques (Molina *et al.*, 2006, 2008). Specifically, CBA/SIV accentuated a loss of SKM mass (SAIDS wasting) and promoted a dysfunctional SKM phenotype, which was associated with accelerated progression to end-stage disease, compared with isocaloric sucrose SIV-infected (SUC/SIV) macaques (Molina *et al.*, 2008). The mechanisms preceding and leading to accentuated SAIDS wasting in CBA/SIV macaques include localized SKM inflammation, increased proteasomal activity, depletion of SKM antioxidant capacity and increased expression of pro-fibrotic genes (LeCapitaine *et al.*, 2011; Dodd *et al.*, 2014). Additionally, using microarray gene analysis, we showed that CBA alters the expression of genes required for mitochondrial function and energy metabolism in the SKM of SIV-infected macaques (Simon *et al.*, 2015).

Muscle protein balance and growth, critical determinants of muscle mass and function, rely on balanced mitochondrial regulation (homeostasis) (Wagatsuma and Sakuma, 2013; Romanello and Sandri, 2016). Impaired mitochondrial biogenesis and respiration has been associated with increased oxidative stress and prevalence of sarcopenia (Richert *et al.*, 2011; Marzetti *et al.*, 2013; Hepple, 2014). At the core of the coordinated network of mitochondrial-related gene regulation are the peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1 family of genes (PGC-1α and PGC-1β). The PGC-1 gene family are master regulators of mitochondrial homeostasis via their activation of genes responsible for mitochondrial DNA replication, oxidative phosphorylation proteins and glucose and fatty acid oxidation [e.g. nuclear respiratory factor 1/2 (NRF-1, NRF-2), PPARs, estrogen-related receptor alpha (ERRα) and mitochondrial transcription factor A (TFAM)] (Kelly and Scarpulla, 2004; Finck and Kelly, 2006). As highly dynamic organelles, mitochondria are continuously undergoing fusion and fission, coordinated by dynamin-1-like protein (DRP1), optic atrophy 1 (OPA1) and mitofusin 1/2 (MFN1, MFN2) (Yin and Cadenas, 2015). This remodeling process requires balance and coordination, and when dysregulated by cellular stress, can lead to cell death signaling either through autophagy or apoptosis (Frank *et al.*, 2001; Pernas and Scorrano, 2016). Mitochondria are important organelles that influence cell death control and generation of reactive oxygen species (ROS). Mitochondrial dyshomeostasis leads to an imbalance in mitochondrial autophagy (mitophagy) and apoptosis. Mitophagy is a compensatory 'quality-control' mechanism that removes damaged mitochondria from the cell, preventing further ROS generation and cell death. Damaged mitochondria undergo DRP1-mediated fission and removal via autophagosome formation coordinated by transcription factor EB (TFEB) and PTEN-induced putative kinase 1 (PINK1; Kubli and Gustafsson, 2012; Hood *et al.*, 2016). With increasing cellular stress, mitophagy fails to protect the cell and apoptotic signaling is initiated through inhibition of anti-apoptotic and pro-survival proteins (Beclin-1 and BCL2) and increased pro-apoptotic signaling proteins (BAX and BAK) (Marino *et al.*, 2014). Excessive ROS generation, defective oxidant scavenging, or both, have been implicated in mitochondrial dysfunction underlying sarcopenia and pathogenesis of several myopathies (Calvani *et al.*, 2013; Lightfoot *et al.*, 2015). Excessive alcohol consumption induces damage to mitochondrial DNA and increases cellular oxidative stress (Krahenbuhl, 2001; Hoek *et al.*, 2002; Bailey, 2003; Bonet-Ponce *et al.*, 2015). Furthermore, HIV infection has been associated with increased permeability of the mitochondrial outer membrane and initiation of apoptotic cell death (Huang *et al.*, 2012). The studies described in this manuscript tested the hypothesis that CBA leads to dysregulation of mitochondrial gene expression in SKM of SIV-infected rhesus macaques at end-stage disease. We determined SKM mitochondrial gene expression central to mitochondrial homeostasis.

MATERIALS AND METHODS

The SKM used for these studies was obtained from animals used in experiments approved by the Institutional Animal Care and Use Committee at both Tulane National Primate Research Center (TNPRC) in Covington, Louisiana and Louisiana State University Health Sciences Center (LSUHSC) in New Orleans, Louisiana, and adhered to National Institutes of Health guidelines for the care and use of experimental animals. Four- to six-year-old male rhesus macaques (*Macaca mulatta*) obtained from TNPRC breeding colonies were used for these studies as previously described (Molina *et al.*, 2008; LeCapitaine *et al.*, 2011).

Experimental protocol

A total of 23 macaques were studied from 3 experimental groups. SKM samples were obtained from a sucrose-administered, SIV-infected group (SUC/SIV, $n = 5$); a CBA-administered, SIV-infected group (CBA/SIV, $n = 6$) and a SIV-negative, healthy macaque control group (CTRL, $n = 12$) used as reference values for comparison of analyzed variables. The cohorts of animals have been used for other studies and results have been previously published (Molina *et al.*, 2008; LeCapitaine *et al.*, 2011; Dodd *et al.*, 2014; Simon *et al.*, 2015). CBA/SIV macaques were administered daily intragastric alcohol at 2.5 g per kg body weight (30% w/v water) or isocaloric sucrose (SUC/SIV) starting 3 months prior to SIV infection (SIV_{mac251}), and continued for the duration of the study. This protocol of intragastric alcohol delivery provided an average of 15% of the animals' total daily caloric intake and produced peak blood alcohol concentrations of 50–60 mM. SKM samples were obtained at necropsy (~14–18 months

post-SIV) when animals met any one of the criteria for euthanasia based on the following: (a) loss of 25% of body weight from maximum body weight since assignment to protocol, (b) major organ failure or medical conditions unresponsive to treatment, (c) surgical complications unresponsive to immediate intervention or (d) complete anorexia for 4 days. All SKM tissue samples were dissected, snap frozen and stored at −80°C until analyses.

SKM gene expression

Total RNA was extracted from SKM using the RNeasy mini universal kit (Qiagen, Valencia, CA) per manufacturer's instructions. cDNA was synthesized from 1 ug of the resulting total RNA using the Quantitect reverse transcriptase kit (Qiagen). Primers were designed to span exon–exon junctions and purchased from IDT (Table 1). The final reactions were made to a total volume of 20 ul with Quantitect SyBr Green polymerase chain reaction (PCR) kit and DNase RNase-free water (Qiagen). All reactions were carried out in duplicate on a CFX96 system (Bio-Rad Laboratories, Hercules, CA) for quantitative PCR (qPCR) detection. qPCR data were analyzed using the comparative Ct (delta–delta-Ct) method. Target genes were compared with the endogenous control, ribosomal protein S13 (RPS13) and experimental group values were normalized to CTRL values.

Statistical analysis

Statistical significance was set at $P \leq 0.05$ and data are displayed as mean ± SEM. Statistical analysis of gene expression was determined

Table 1. qPCR primers (*M. mulatta*)

Target gene	5′ to 3′	Primer sequences
Autophagy related 5 (ATG5)	Forward	ACCAGAAACACTTCGCTGCT
	Reverse	ATGATGGCAGTGGAGGAAAG
Beclin-1	Forward	AGCTTTTGTCCACTGCTCCTC
	Reverse	GGTTGAGAAAGGCGAGACA
B-cell lymphoma 2 (BCL2)	Forward	GGCCAGGGTCAGAGTTAAATA
	Reverse	GAGGTTCTCGGATGTTCTTCTC
BCL2 associated agonist of cell death (BAD)	Forward	TGGTGGGATCGGAACTT
	Reverse	TCCGCCCATATCCAAGAT
BCL2 antagonist/killer (BAK)	Forward	CCATGCTGGAGTGAGAATAAA
	Reverse	TTCCAAAGTGCTGGGATTAC
BCL2 associated X protein (BAX)	Forward	GAGCTGCAGAGGATGATTG
	Reverse	GCCTTGAGCACCAGTTT
Caspase-9	Forward	GAGGAAGAGGGACAGATGAATG
	Reverse	AGGTTAATCCCTGCCCTAGA
DRP1	Forward	TCCTCTCCCATCCATCTTATC
	Reverse	TTCTTGTACTCCTCCACCTC
ERRα	Forward	TGCACTGGTGTCTCATCT
	Reverse	GAAACCTGGGATGCTCTTG
Glutathione synthetase (GSS)	Forward	CGGACAGTGAGATGTAGGAAAG
	Reverse	GAGTCTCCACACAACCAGAATAG
MFN1	Forward	CTCCAGCAACACCAGATAAT
	Reverse	GTCCAGGACAGTCTTTCATAC
MFN2	Forward	CAGGAGGAGTTCATGGTTTC
	Reverse	AGACGCTCATAGACGTAGAG
NRF-1	Forward	CACGCACAGTATAGCTCATC
	Reverse	GTAGCCCTCAAGTTTACTCAC
NRF-2	Forward	CTACTTGGCCTCAGTGATTC
	Reverse	R-GACAAGGGTTGTACCGTATC
OPA1	Forward	GGACAGCTTGAGGGTTATTC
	Reverse	GTTCTTGGGTCCGATTCTTC
PPARα	Forward	CCTGCGAACATGACATAGAA
	Reverse	CCATACACAGTGTCTCCATATC
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)	Forward	TGAACTGAGGGACAGTGATTTC
	Reverse	CCCAAGGGTAGCTCAGTTTATC
Peroxisome proliferator-activated receptor gamma coactivator 1-beta (PGC-1β)	Forward	GACAAGGCCCTTCCAATATG
	Reverse	CAGCAGTTTCAAGTCTCTCTC
PINK1	Forward	ACAGGCTCACAGAGAAGT
	Reverse	CGTTTCACACTCCAGGTTAG
Ribosomal protein S13 (RPS13)	Forward	TCTGACGACGTGAAGGAGCAGATT
	Reverse	TCTCTCAGGATCACACCGATTTGT
Superoxide dismutase 2 (SOD2)	Forward	GACAAACCTCAGCCCTAATG
	Reverse	CCGTCAGCTTCTCCTTAAAC
TFAM	Forward	CAGCTAACTCCAGATGAGATTAC
	Reverse	GTGATTCACCCTTAGCTTCTT
TFEB	Forward	GCAACAGTGCTCCCAATA
	Reverse	GACATCATCAAACTCCCTCTC

using one-way ANOVA. Fisher's least significant difference *post hoc* test was used to determine pairwise differences. Statistical analyses were performed using SPSS (IBM SPSS Statistics version 22, Chicago, IL).

RESULTS

CBA dysregulates mitochondrial-related gene expression in the SKM of SIV-infected macaques at end stage

SIV infection resulted in significant upregulation of SKM expression of NRF-1, mitofusin 2 (MFN2) and glutathione synthetase (GSS) compared to uninfected controls. CBA resulted in significant downregulation of peroxisome proliferator-activated receptor-γ coactivator (PGC)-1ß compared to uninfected controls and prevented the SIV-induced upregulation of GSS. Moreover, CBA resulted in significant upregulation of ERRα, NRF-1 and superoxide dismutase 2 (SOD2) compared to uninfected controls (Fig. 1). There were no differences between groups for PGC-1α, NRF-2 or TFAM expression. Gene expression of mitochondrial dynamin like GTPase (OPA1) and MFN1 was below the level of detection.

CBA prevented mitophagy-related gene expression in the SKM of SIV-infected macaques at end stage

SIV infection resulted in a significant upregulation of mitophagy-related gene expression including DRP1, TFEB and PINK1. CBA prevented the SIV-induced increased expression of TFEB and PINK1 (Fig. 2).

CBA increases apoptosis-related gene expression in the SKM of SIV-infected macaques at end stage

SIV infection resulted in significant upregulation of BCL2 antagonist/killer (BAK) without altering that of Beclin-1, B-cell lymphoma 2 (BCL2), BCL2 associated X protein (BAX) or Caspase-9. CBA resulted in significant suppression of Beclin-1 and BCL2 compared to uninfected controls. CBA resulted in a significant reduction of Caspase-9 compared to SUC/SIV. In contrast, CBA resulted in marked upregulation of BAX and BAK expression compared to uninfected controls (Fig. 3). No significant differences were detected between groups for BAD or ATG5 expression.

DISCUSSION

This study examined the effects of CBA on expression of mitochondria-related genes in SKM of non-ART treated SIV-infected male rhesus

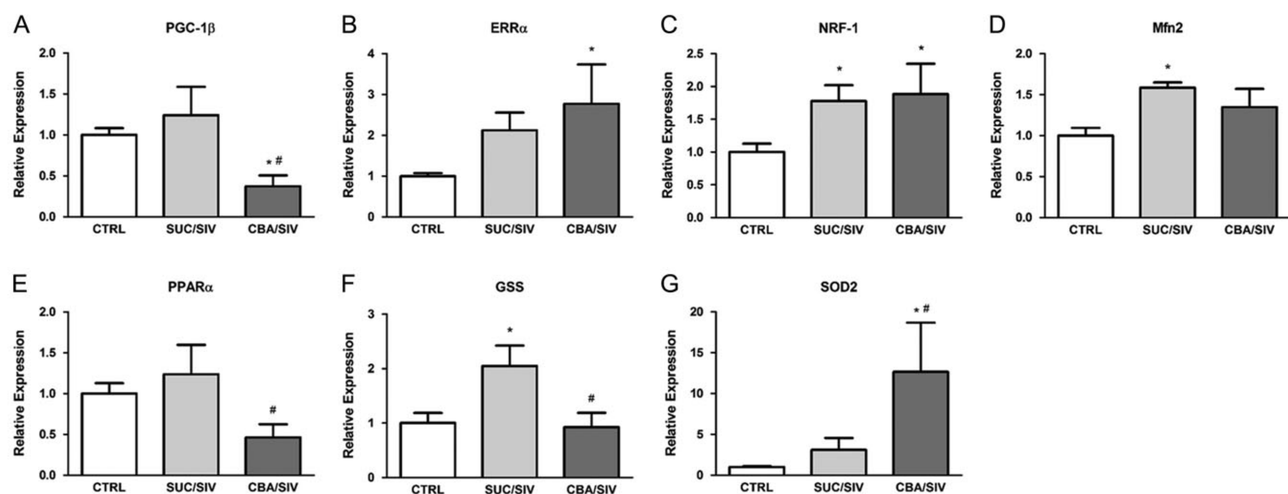

Fig. 1. Mitochondrial-related gene expression. PGC-1β (**A**) expression was significantly decreased in SKM of CBA/SIV macaques compared to SUC/SIV and CTRL. ERRα (**B**) and NRF-1 (**C**) were significantly increased in SKM of CBA/SIV macaques compared to CTRL. PPARα (**E**) and GSS (**F**) expression was significantly lower in SKM of CBA/SIV compared to SUC/SIV. SOD2 (**G**) expression was significantly greater in SKM of CBA/SIV macaques compared to SUC/SIV and CTRL. The SKM of SUC/SIV had significantly increased expression of NRF-1, MFN2 (**D**) and GSS compared to CTRL. *Significant ($P < 0.05$) difference from CTRL. #Significant ($P < 0.05$) difference between CBA/SIV and SUC/SIV. Data are expressed as mean ± SEM.

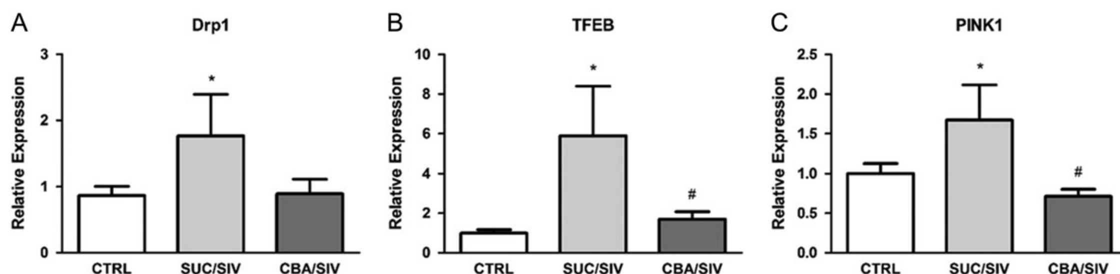

Fig. 2. Mitophagy-related gene expression. DRP1 (**A**), TFEB (**B**) and PINK1 (**C**) expression was significantly greater in SKM of SUC/SIV compared to CTRL. TFEB and PINK1 expression was significantly lower in SKM of CBA/SIV compared to SKM of SUC/SIV macaques. *Significant ($P < 0.05$) difference from CTRL. #Significant ($P < 0.05$) difference between CBA/SIV and SUC/SIV. Data are expressed as mean ± SEM.

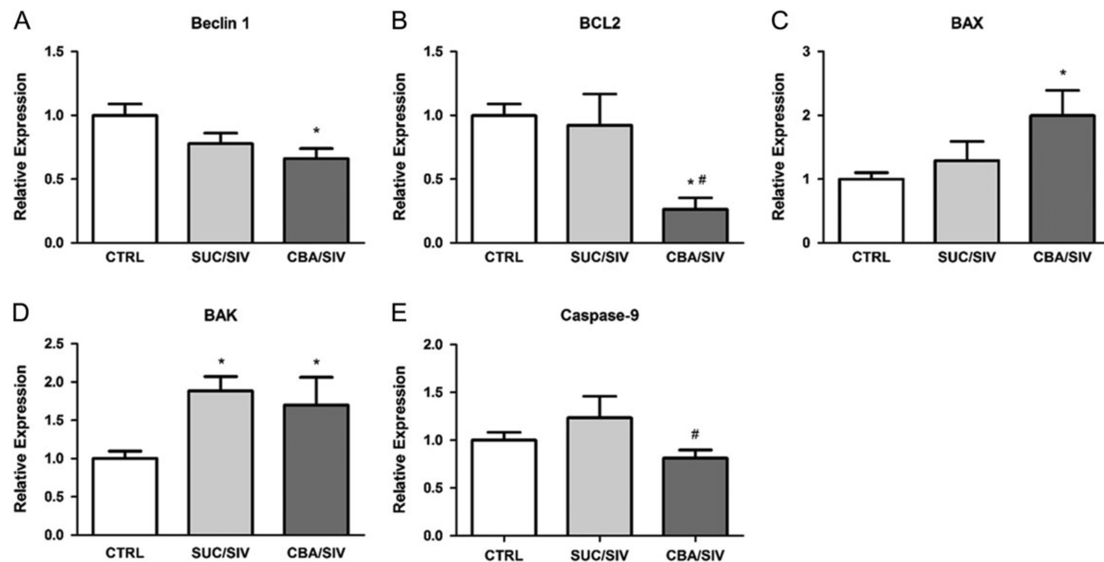

Fig. 3. Apoptosis-related gene expression. Beclin-1 (**A**) expression was significantly lower in CBA/SIV compared to CTRL. BCL2 (**B**) expression was significantly lower in CBA/SIV SKM compared to that in SUC/SIV and CTRL. BAX (**C**) and BAK (**D**) expression was significantly greater in SKM of CBA/SIV compared to SKM of SUC/SIV and CTRL. Caspase-9 (**E**) was lower in CBA/SIV compared to SUC/SIV. *Significant ($P < 0.05$) difference from CTRL. #Significant ($P < 0.05$) difference between CBA/SIV and SUC/SIV. Data are expressed as mean ± SEM.

macaques at end-stage disease. SIV infection was associated with increased expression of NRF-1, MFN2, GSS and mitophagy-related genes compared to uninfected controls. CBA resulted in downregulation of PGC-1β and upregulation of ERRa, NRF-1 and SOD2 compared to uninfected controls. CBA prevented the SIV-induced upregulation of GSS and mitophagy-related gene expression. Additionally, CBA resulted in suppression of anti-apoptotic gene expression and upregulation of pro-apoptotic gene expression. These findings suggest that SIV infection disrupts mitochondrial homeostasis and triggers compensatory mitophagy and antioxidant gene upregulation. When combined with CBA (CBA/SIV), SKM showed differential expression of genes involved in apoptotic signaling, which could potentially contribute to the underlying pathophysiology of CBA-induced accelerated SKM wasting at end-stage SIV disease (Fig. 4).

Mitohormesis, the adaptive response to disruptions to the dynamic balance of mitochondrial signaling, increases cellular tolerance to oxidative stressors (Barbieri *et al*., 2014). Low-level oxidative stress (ROS) can signal a mitohormetic response, resulting in improved mitochondrial functioning (e.g. adenosine triphosphate production and antioxidant capacity). Canonically, this is achieved through chronic exercise training (Barbieri *et al*., 2014; Merry and Ristow, 2016) and represents an advantageous response to oxidative stress in the cell. However, levels of ROS that exceed the mitochondria's ability to maintain homeostasis have been implicated as a major factor in several metabolic diseases (Nunnari and Suomalainen, 2012). Previous studies from our laboratory reported that SIV infection resulted in a 2.5-fold reduction in SKM antioxidant capacity and this was further decreased (~7-fold reduction) by CBA (LeCapitaine *et al*., 2011), suggesting an alcohol-induced upregulation in ROS production. This is supported by the observed expression of genes related to antioxidant signaling in the present study. We found that CBA prevented an increase in GSS, a precursor to glutathione and increased SOD2 expression. The increase in SOD2 expression in CBA/SIV macaques suggests increased presence of the superoxide anion (Kowaltowski *et al*., 2009) and is in agreement with our previous findings of decreased antioxidant capacity in muscle of CBA/SIV macaques (LeCapitaine *et al*., 2011). In the present study, CBA caused a reduction in PGC-1β expression compared to uninfected controls and SUC/SIV. PGC-1β is a member of the PPARγ coactivator-1 family of genes (PGC-1α and PGC-1β), master regulators of mitochondrial homeostasis and important mediators of mitochondrial biogenesis and expression of oxidative phosphorylation proteins (Kelly and Scarpulla, 2004; Finck and Kelly, 2006). PGC-1β deficiency in SKM has been reported to result in abnormal mitochondrial structure, reduced antioxidant defense and increased oxidative stress (Gali Ramamoorthy *et al*., 2015). Thus, our findings suggest that compensatory mitochondrial biogenesis is likely impaired in muscle of CBA/SIV macaques and may be an important mechanism contributing to previously reported pro-oxidative and catabolic milieu.

Cellular stressors, such as increased ROS production, initiate signal transduction required for mitophagy. This adaptive response removes damaged mitochondria which could otherwise contribute to further escalation in ROS generation (Marino *et al*., 2014). Our results show an SIV-induced increase of genes related to mitophagy signaling that was partially prevented by CBA. Autophagy pathways precede apoptosis, can inhibit apoptotic signaling and often represent an avoidance of cell death (Marino *et al*., 2014). Under extraordinary cellular stress conditions, autophagy signaling (including mitophagy) pathways are inhibited and pro-apoptotic signaling activated. In many tissues, caspase activation is highly associated with apoptosis. However, caspase-independent apoptosis, involving the release of cell-toxic proteins (e.g. EndoG and AIF), has been implicated as an important factor in muscle atrophy (Dupont-Versteegden, 2006). These pro-apoptotic proteins are able to translocate into the nucleus, causing damage to DNA, and ultimately cell death (Dupont-Versteegden, 2006). Central to the initiation of this apoptotic cascade is the activation of BAX and BAK, which allow the permeabilization of the outer mitochondrial membrane and release of pro-apoptotic proteins (Chipuk *et al*., 2010). This permeabilization represents a 'point of no return' and, thus, a critical component of cell death signaling. In the present study, we found that anti-apoptotic genes Beclin-1 and BCL2 were decreased

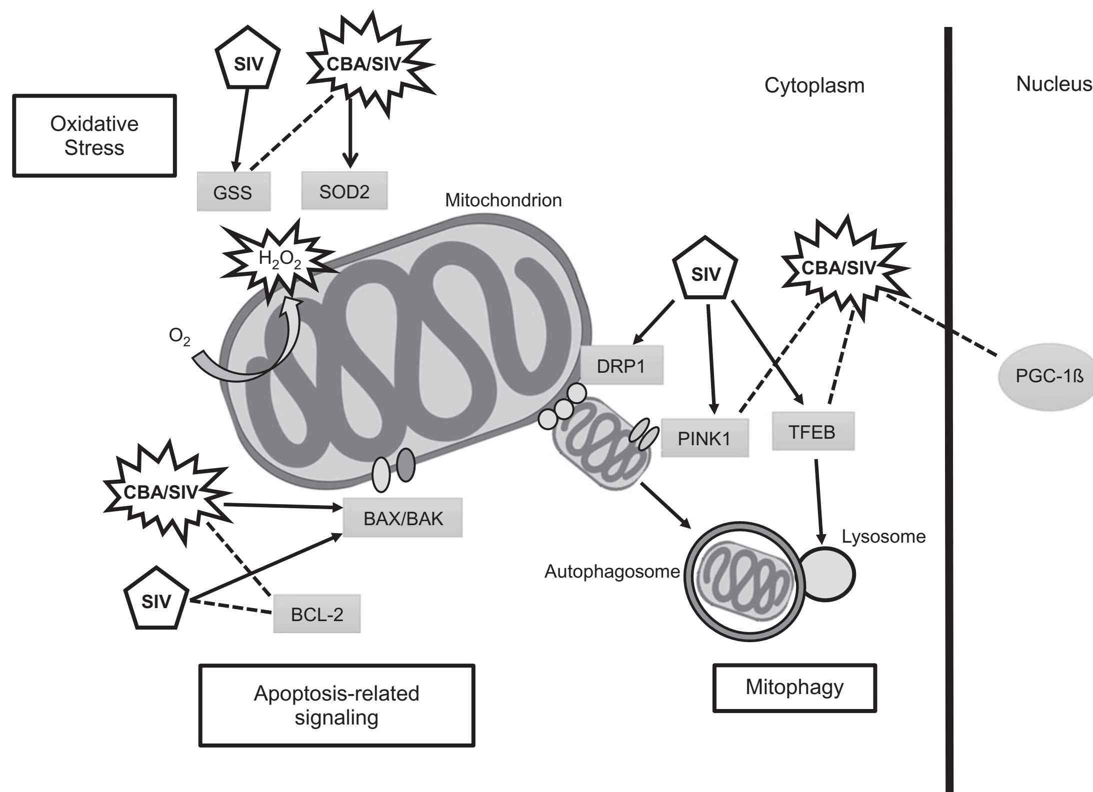

Fig. 4. Pictorial summary of main SIV and CBA effects on SKM gene expression and their potential impact on regulation mitochondrial homeostasis. Three main pathways are predicted to be impacted by the observed alterations in gene expression in addition to the overall CBA/SIV-induced decreased expression of PGC-1ß, a master regulator of mitochondrial homeostasis; mitophagy, apoptosis and oxidative stress. SIV increased expression of mitophagy-related genes (DRP1, PINK1 and TFEB), while CBA/SIV prevented the increase of PINK1 and TFEB. DRP1 regulates the fission of damaged mitochondria and initiates mitophagy. As the outer mitochondrial membrane becomes dysfunctional PINK1 is activated and contributes the formation of an autophagosome. TFEB is an activator of lysosomal biogenesis which allows the fusing of the autophagosome with lysosomes for mitophagy. SIV and CBA/SIV decreased expression of the anti-apoptotic gene BCL2. CBA/SIV upregulated both BAX and BAK gene expression. Apoptotic-related signaling is initiated in response to increased cellular stress and permeabilization (BAX/BAK) of the outer mitochondrial membrane. SIV increased expression of GSS. CBA/SIV prevented the increase in GSS while increasing SOD2 gene expression. Damaged mitochondria generate increased levels of the ROS superoxide, which is converted to H_2O_2 by SOD2. GSS is a precursor to glutathione, which contributes to scavenging of H_2O_2. These results suggest that SIV infection disrupts mitochondrial homeostasis and when combined with CBA, results in differential expression of genes involved in mitophagy, apoptosis and anti-oxidative capacity.

and pro-apoptotic genes BAX and BAK were increased in SKM of CBA/SIV. These findings are supportive to our hypothesis that CBA-induced mitochondrial dysregulation may contribute to metabolic dysfunction and accentuated muscle wasting in SIV-infected macaques.

The current study demonstrates CBA-induced mitochondrial gene dysregulation in non-ART treated SIV-infected macaques at end-stage disease. Limitations of this study include the lack of mechanistic experiments to elucidate the functional relevance of dysregulated mitochondrial gene expression and analysis of protein expression. Future studies will utilize *in vitro* models to analyze protein abundance and mechanistically investigate chronic alcohol-induced mitophagy/apoptosis signaling and how their imbalance contributes to SKM wasting.

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CONFLICT OF INTEREST STATEMENT

None declared.

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